Cryptococcus gattii and *Cryptococcus neoformans* - Cosmopolitans on the move

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Cover design: the electron micrograph of *Cryptococcus neoformans* var. *grubii* strain CBS1009 was made by Jan Dijksterhuis. This strain was isolated in 1934 by the neurologist Hendrik W. Stenvers (Utrecht) who described the first cryptococcal infection in the Netherlands. The original drawings by his hand were used in a modified format with permission from the publisher (Stenvers HW. *Torula* infectie van het centrale zenuwstelsel. Ned Tijdschr Geneeskd. 1934;78:3361-3366).

For the invitation card a modified scanning electron micrograph of the teleomorph *Filobasidiella neoformans* (6,000× magnification) was used (courtesy of Rob Samson).

Cryptococcus gattii and *Cryptococcus neoformans*
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Cosmopolitans on the move

Cryptococcus gattii* en *Cryptococcus neoformans
–
Wereldreizigers in beweging

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 25 oktober 2011 des middags te 2.30 uur

door

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geboren op 27 februari 1979 te Hilversum

Promotor: Prof. dr. I.M. Hoepelman

Co-promotor: Dr. T. Boekhout

Voor mijn familie

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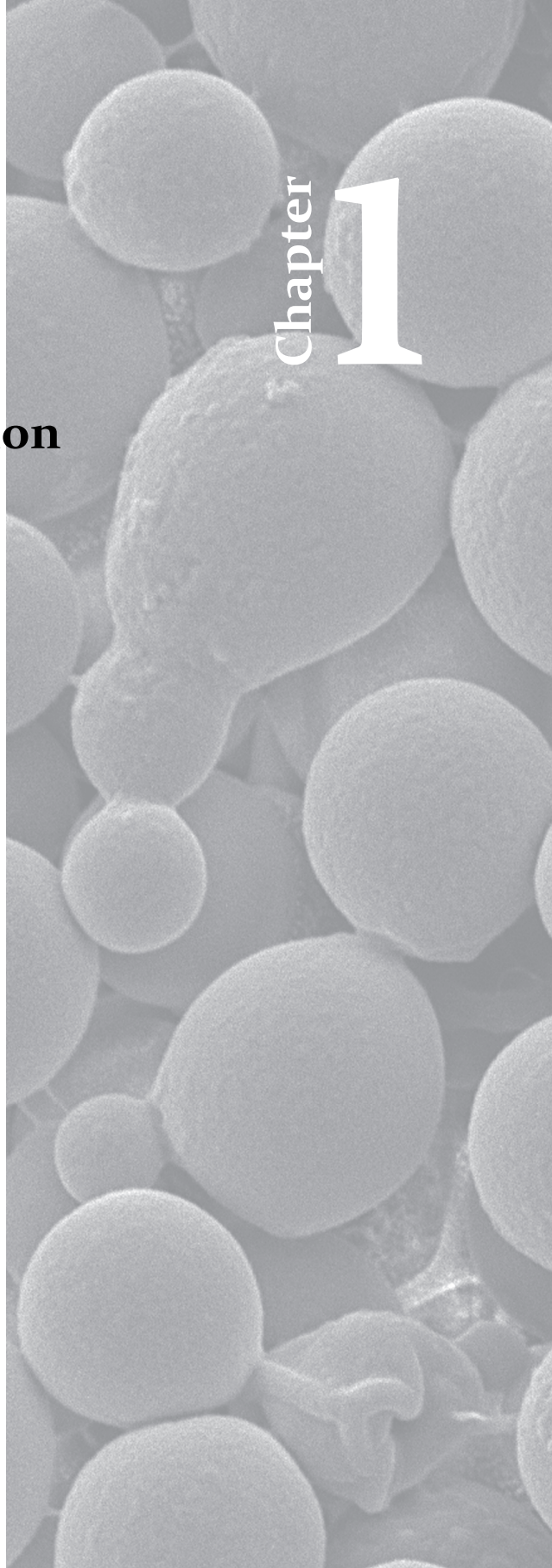

Chapter 1

General Introduction

Introduction

The fungal kingdom is the second largest group in the domain of eukaryotic organisms. Only the kingdom of insects has a higher predicted species richness than the estimated 1.5 million fungal species (Hawksworth, 1991; Mueller and Schmit, 2007). At present, roughly over 100.000 fungal species have been described, which includes a number of amply 1.500 unicellular species, the yeasts (Boekhout, 2005; Kurtzman *et al.*, 2011; Mueller & Schmit, 2007). The majority of fungi do not cause any harm to living organisms and some fungi are beneficial to mankind since they can be exploited for food, drug and industrial applications (Boekhout, 2005; Nielsen & Heitman, 2007). However, the number of fungal species that frequently cause disease in humans has rapidly expanded since the onset of the HIV-pandemic that emerged thirty years ago (Nielsen & Heitman, 2007). Approximately 0.3% of the currently recognized fungal species is known to cause infections in humans (Woolhouse & Gaunt, 2007). Only a handful of these pathogenic fungal species can be regarded as a major cause of morbidity and mortality among immunocompromised individuals (Ampel, 1996; Walsh & Groll, 1999). The most prevalent occurring human pathogenic fungi are *Candida* species that are a major cause of nosocomial blood stream infections, *Aspergillus* species that are a frequent cause of mortality in patients that underwent bone marrow transplantation, and *Cryptococcus neoformans*, which is the major cause of meningoencephalitis among HIV-infected individuals (Mitchell & Perfect, 1995; Walsh & Groll, 1999; Warkentien & Crum-Cianflone, 2010).

A first attempt to estimate the global impact on human health by a fungal pathogen was carried out for *C. neoformans* and showed that annually approximately one million individuals develop cryptococcal meningitis (Park *et al.*, 2009). Cryptococcal infections accounted yearly for an estimated number of nearly 625.000 fatalities, mainly in sub-Saharan Africa where a relatively high incidence of HIV-infections placed cryptococcal meningitis at the fourth position as a HIV-related cause of dead (Park *et al.*, 2009; Warkentien & Crum-Cianflone, 2010).

Not only immunocompromised patients, but also immunocompetent individuals are threatened by fungal pathogens. This has been demonstrated by an expanding outbreak of the primary pathogenic yeast *Cryptococcus gattii* that has caused serious disease in hundreds of people and animals (Byrnes *et al.*, 2010; Datta *et al.*, 2009; Kidd *et al.*, 2004).

Taxonomic history of *Cryptococcus*

Cryptococcus neoformans has become one of the most prevalent causes of fungal disease. Untill the early 1980s the species was regarded as a rare cause of infection among immunocompromised and immunocompetent individuals (Mitchell & Perfect, 1995). The first description of a cryptococcal infection dates back to 1894 when the German physicians Otto Busse and Abraham Buschke cultivated the yeast *Saccharomyces hominis* from a lesion of a women's tibia (Barnett, 2010; Buschke, 1895; Busse, 1894).

In the same year, Francesco Sanfelice, an Italian researcher, found similar yeast cells in fermenting peach juice and proved that the obtained yeast was pathogenic when injected in laboratory animals (Barnett, 2010; Sanfelice, 1894). In 1901, the French mycologist Jean-Paul Vuillemin discovered that the *S. hominis* isolates were unable to produce ascospores and placed them in the genus *Cryptococcus*. Based on the morphological characteristics, Vuillemin decided to rename the clinical isolate from Busse and Buschke to *Cryptococcus hominis* and the environmental isolate cultured by Sanfelice as *Cryptococcus neoformans* (Barnett, 2010; Vuillemin, 1901). The type-strain of *C. hominis* was not preserved and thus became *C. neoformans* the valid species name. During the first decades of the 20th century, several case reports described infections similar to cryptococcosis but introducing new synonyms for the same infectious microorganism: *C. neoformans*. This has led to an extensive list of nearly fifty synonyms as listed in Table 1 (Barnett *et al.*, 2010; Bovers *et al.*, 2008a).

In the 1950s, it was found that *C. neoformans* could be divided in three groups based on capsular antigen properties and these were designated serotypes A, B and C. The fourth serotype, logically named serotype D, was identified nearly two decades thereafter (Evans *et al.*, 1950; Wilson *et al.*, 1968). During the 1970s, the sexual phase of *C. neoformans* was described and the opposite mating-types were named **a** and α (Kwon-Chung, 1975). It was observed that the sexual state of serotype A and D isolates differed microscopically from that of serotype B and C isolates. Therefore, both groups where accommodated in the genus *Filobasidiella* as *F. neoformans* for serotype A and D isolates and *F. bacillispora* for serotype B and C isolates (Kwon-Chung, 1976a, 1976b). The latter species was a few years later reduced to a variety of *F. neoformans* as *F. neoformans* var. *bacillispora*. Since then *C. neoformans* was split into *C. neoformans* var. *neoformans* (serotype A and D) with the teleomorphic phase *F. neoformans* var. *neoformans*, and *C. neoformans* var. *gattii* (serotype B and C) with the teleomorphic phase *F. neoformans* var. *bacillispora* (Kwon-Chung *et al.*, 1982a). The proposal to divide the two serotypic groups into two taxonomic varieties was further strengthened by biochemical characteristics and the ecological and geographical differences between serotype A and D versus serotype B and C isolates (Bennett *et al.*, 1977, 1978; Kwon-Chung & Bennett, 1984; Vanbreuseghem & Takashio, 1970).

The last major changes in the taxonomy of *C. neoformans* was the proposal to raise the group of serotype A isolates to a separate variety level named *C. neoformans* var. *grubii* (Franzot *et al.*, 1999). This was based on the distinct phylogeny, epidemiological and biochemical differences, when compared to serotype D isolates that were represented by the novel variety *C. neoformans* var. *neoformans* (Franzot *et al.*, 1999). Finally, phylogenetic analyses, biochemical, ecological and epidemiological differences were the major reasons to raise *C. neoformans* var. *gattii* to the species level named *C. gattii*, a proposal that was immediately embraced by the cryptococcal research community (Kwon-Chung *et al.*, 2002). As a consequence, the teleomorphic species name *F. bacillispora* was reintroduced for the sexual phase of *F. neoformans* var. *bacillispora* (Kwon-Chung *et al.*, 2002).

The advance of molecular biological techniques in the field of medical mycology (see below) has clearly shown that the current taxonomy of *C. neoformans*

Table 1. Taxonomic history of *Cryptococcus neoformans* and *Cryptococcus gattii*

Year	Synonym	Remark (^{T} = type-strain)	Reference
1895	*Saccharomyces neoformans*	Isolated from fruit juice, CBS132^T	Sanfelice, 1895a
1895	*Saccharomyces litogenes*		Sanfelice, 1895b
1895	*Megelococcus myxoides*		Curtis, 1895
1896	*Saccharomyces subcutaneous tumefaciens*	CBS1622^T	Curtis, 1896
1901	*Cryptococcus hominis*		Vuillemin, 1901
1901	*Cryptococcus lithogenes*	Replacing *S. litogenes*	Vuillemin, 1901
1901	*Cryptococcus neoformans*	Replacing *S. neoformans*; CBS132^T	Vuillemin, 1901
1901	*Saccharomyces hominis*	CBS879^T	Costantin, 1901
1901	*Saccharomyces plimmeri*		Costantin, 1901
1902	*Torula klein*		Weis, 1902
1902	*Torula neoformans*	Original strain of *S. neoformans*; CBS132^T	Weis, 1902
1902	*Torula plimmeri*		Weis, 1902
1904	*Cryptococcus kleinii*		Guéguen, 1904
1908	*Cryptococcus plimmeri*		Neveu-Lemaire, 1908
1909	*Atelosaccharomyces busse-buschki*		de Beurmann & Gougerot, 1909
1910	*Saccharomyces blanchardii*		Guiart, 1910
1912	*Cryptococcus gotti*		Neveu-Lemaire, 1912
1912	*Cryptococcus guilliermondii*		Beauverie & Lesieur, 1912
1913	*Atelosaccharomyces breweri*		Verdun, 1913
1913	*Atelosaccharomyces hominis*	Synonym of *C. hominis*	Verdun, 1913
1916	*Torula histolytica*		Stoddard & Cutler, 1916

Table 1. *continued*

1918	*Cryptococcus costantini*		Fernandes & Froilano de Mello, 1918
1919	*Cryptococcus breweri*	Synonym of *C. breweri*	Castellani & Chalmers, 1919
1921	*Saccharomyces breweri*	Synonym of *A. breweri*	Neveu-Lemaire, 1921
1922	*Blastomyces lithogenes*		Sasakawa, 1922
1927	*Cryptococcus histolyticus*	Name change of *Torula histolytica*	Castellani, 1927
1928	*Torula nasalis*	CBS882^T	Harrison, 1928
1930	*Cryptococcus psicrofilicus*	Description and drawing of yeast cells; CBS996^T	Speroni *et al.*, 1930
1931	*Cryptococcus cerebriloculosis*		Freeman, 1931
1931	*Torulopsis hominis*	Synonym of *C. hominis*	Redaelli, 1931
1933	*Torulopsis breweri*	Synonym of *A. breweri*, *C. breweri* and *S. breweri*	de Almeida, 1933
1933	*Torulopsis costantini*	Synonym of C. costantini	de Almeida, 1933
1933	*Torulopsis lithogenes*	Synonym of *B. lithogenes*, *C. lithogenes* and *S. lithogenes*	de Almeida, 1933
1933	*Torulopsis plimmeri*	Synonym of *C. plimmeri* and *S. plimmeri*	de Almeida, 1933
1933	*Cryptococcus hondurianus*	CBS883^T	Castellani, 1933
1933	*Torulopsis histolytica*		Castellani & Jacono, 1933
1934	*Blastomyces neoformans*	Considered to be *C. hominis* and *S. neoformans*	Lodder, 1934
1934	*Torulopsis neoformans*	Considered to be *C. hominis* and *S. neoformans*	Lodder, 1934
1935	*Cryptococcus meningitidis*	Synonym of *Torula histolytica*	Dodge, 1935
1935	*Cryptococcus nasalis*	Synonym of *Torula nasalis*	Dodge, 1935
1935	*Torulopsis neoformans* var. *sheppei*	CBS919^T	Giordano, 1936
1936	*Debaryomyces hominis*		Todd & Hermann, 1935
1937	*Debaryomyces neoformans*		Redaelli *et al.*, 1937

Table 1. *continued*

1954	*Rhodotorula histolytica*	Synonym of *Torula histolytica*	Kursanov *et al.*, 1954
1954	*Torulopsis nasalis*	Synonym of *Torula nasalis*	Kursanov *et al.*, 1954
1960	*Lipomyces neoformans*		Ciferrim, 1960
1970	*Cryptococcus neoformans* var. *gattii*	CBS6289^{T}	Vanbreuseghem & Takashio, 1970
1975	*Filobasidiella neoformans*	Teleomorph of *C. neoformans* var. *neoformans*	Kwon-Chung, 1975
1976	*Filobasidiella bacillispora*	Teleomorph of *C. neoformans* var. *gattii*	Kwon-Chung, 1976b
1978	*Cryptococcus bacillisporus*	Synonym of *C. neoformans* var. *gattii*; CBS6955^{T}	Kwon-Chung *et al.*, 1978
1982	*Cryptococcus neoformans* var. *gattii*	*C. neoformans* serotype B and C; CBS6289^{T}	Kwon-Chung *et al.*, 1982a
1982	*Cryptococcus neoformans* var. *neoformans*	*C. neoformans* serotype A and D	Kwon-Chung *et al.*, 1982a
1983	*Cryptococcus shanghaiensis*	CBS7229^{T}	Liao *et al.*, 1983
1999	*Cryptococcus neoformans* var. *grubii*	*C. neoformans* serotype A isolates; CBS8710^{T}	Franzot *et al.*, 1999
1999	*Cryptococcus neoformans* var. *neoformans*	*C. neoformans* serotype D isolates; CBS882^{T}	Franzot *et al.*, 1999
2002	*Cryptococcus gattii*	Replaces *C. neoformans* var. *gattii*; CBS6289^{T}	Kwon-Chung *et al.*, 2002

and *C. gattii* needs a thorough revision. Again, epidemiological and phylogenetic differences between the different genetic groups within both *Cryptococcus* species play an important role in the proposals to be made to raise varieties to species level in the future and, probably, to recognize genotypes as varieties (Bovers *et al.*, 2008a, 2008b; D'Souza *et al.*, 2011; Kwon-Chung & Varma, 2006; Ngamskulrungroj *et al.*, 2009).

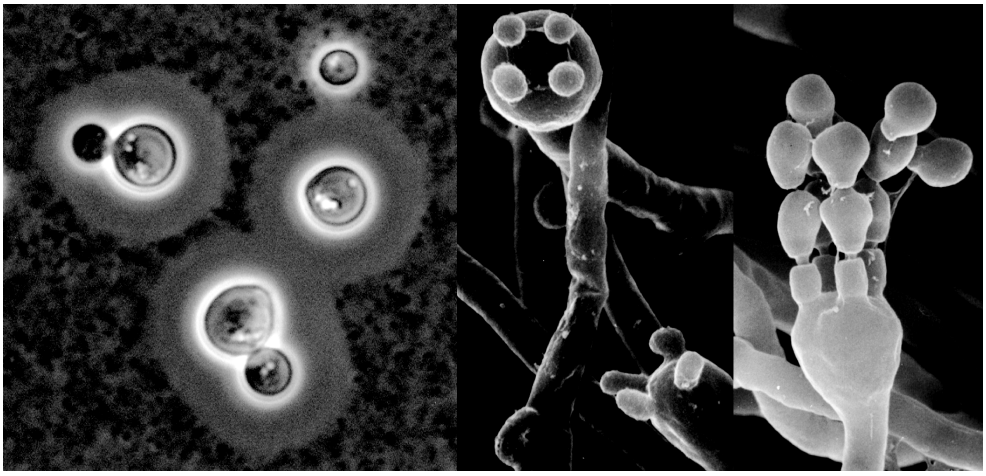

Figure 1. The left panel shows a 1000× magnification of a negative Indian ink staining of *C. gattii* strain CBS883 which reveals its large polysaccharide capsule that is as thick as the diameter of the cell itself (4-6 μm). The center and right panel show scanning electron microscopy photographs of the teleomorphic phase of *C. neoformans* (*Filobasidiella neoformans*) (courtesy of Rob Samson; Samson *et al*., 1983). A clamp connection, a connection between adjacent hyphal cells, is present at the left bottom of the center photograph, as well as two basidia with four basidiospores each. A basidium with four chains of basidiospores is shown in the right photograph.

Conventional identification

Microscopically, yeast cells of *C. neoformans* and *C. gattii* are hardly to differentiate from each other since their yeast cells are both globose to ovoidal. A shared and major characteristic of both species is their thick polysaccharide capsule (Figure 1; Kwon-Chung *et al.*, 1982a). Differences between *C. neoformans* and *C. gattii* become visible when it enters the teleomorphic phase (Kwon-Chung *et al.*, 1975, 1976a, 1976b). However, differentiation can only be made between *C. gattii* versus *C. neoformans* var. *grubii* and var. *neoformans*. During the teleomorphic phase the latter varieties (*F. neoformans*) produce spherical to cylindrical basidiospores which are 1.8-2.5 μm in diameter and have fine roughened walls, while basidiospores of *F. bacillispora*, the teleomorph of *C. gattii*, are thin-walled and narrow to rod-shaped (1.0-1.5 × 3-8 μm) (Figure 1; Franzot *et al.*, 1999; Kwon-Chung, 1975, 1976a, 1976b; Kwon-Chung *et al.*, 2002).

Several media-based differentia-tion methods have been developed to distinguish *C. neoformans* from *C. gattii* and use the property that the latter is

Table 2. Phenotypic and genotypic classification of *Cryptococcus neoformans* and *Cryptococcus gattii*

Phenotypically		DNA fingerprinting based characterization					Sequence based	
CGB/CDBT[1]	Sero-type[2]	AFLP[3]	M13[4] PLB1 RFLP[5]	*URA5* RFLP[6]	$(GACA)_4$[7]	*CAP1+GEF1* RFLP[8]	ITS[9]	IGS[10]
C. neoformans* var. *grubii								
		AFLP1	VNI	A1	VN5 / VN6	C1+G1 (αA) / C2+G2 (**a**A)	ITS1	1A, B
- / M=gold & C=pale	A	AFLP1A/VNB	VNII	A2	nd	C1+G15 (αA) / C15+G2 (**a**A)	ITS1	1C
		AFLP1B	VNII	A2	VN7	C3+G3 (αA)	ITS1	1A
C. neoformans* var. *neoformans								
- / M=orange & C=bright red	D	AFLP2	VNIV	A4	VN1 / VN2	C6+G6 (αD) / C7+G7 (**a**D)	ITS2	2A-C
C. neoformans* var. *neoformans* × *C. neoformans* var. *neoformans								
- / nd	AD	AFLP3	VNIII	A3	VN3 / VN4	C4+G4 (αA**a**D) / C5+G5 (αD**a**A)	ITS2	2C
C. gattii								
+ / M=green & C=dull red	B	AFLP4	VGI	A5	nd	C8+G8 (αB) / C9+G9 (**a**B)	ITS3, 7	4
+ / M=green & C=pale	C	AFLP5	VGIII	A7	nd	C12+G12(αC) / C13+G13 (**a**C)	ITS5	5
+ / M=green & C=dull red	B	AFLP6A	VGIIa	A6	nd	C10+G10 (αB) / C11+G11 (**a**B)	ITS4	3
+ / M=green & C=dull red	B	AFLP6B	VGIIb	A6	nd	C10+G10 (αB) / C11+G11 (**a**B)	ITS4	3
+ / M=green & C=dull red	B	AFLP6C	VGIIc	A6	nd	C10+G10 (αB) / C11+G11 (**a**B)	ITS4	3
+ / M=green & C=pale	C	AFLP7	VGIV	A8	nd	C14+G14 (αC)	ITS6	6
+ / M=green & C=dull red	B	AFLP10	VGIV	A8	nd	nd	nd	nd
C. neoformans* var. *neoformans* × *C. gattii								
w / nd	BD	AFLP8	nd	nd	nd	nd	nd	nd
C. neoformans* var. *grubii* × *C. gattii								
w / nd	AB	AFLP9	nd	nd	nd	nd	nd	nd

resistant or less susceptible to L-canavanine or cycloheximide (Irokanulo *et al.*, 1994; Kwon-Chung *et al.*, 1982b; Salkin & Hurd, 1982). The method based on L-canavanine-glycine-bromothymol blue (CGB) medium is more accurate when compared to glycine-cycloheximide-phenol red or creatinine-dextrose-bromothymol blue media (Table 2; Kwon-Chung *et al.*, 1982b). The latter has been further improved by the addition of thymine which allows further differentiation between the different serotypes of *C. neoformans* and *C. gattii*, based on the color of the media and the colonies (Table 2; Irokanulo *et al.*, 1994). This improved medium seems to be neglected since the CGB medium is more widely used in research settings and routine laboraties, despite the observation that false positive and negative results can occur (Klein *et al.*, 2009; McTaggart *et al.*, 2011; Kwon-Chung *et al.*, 1982b).

Based on the polysaccharide capsule cryptococcal isolates were grouped into serotypes. Serotype A and D represent *C. neoformans* var. *grubii* and var. *neoformans*, respectively, while serotype B and C represent *C. gattii* (Table 2; Kwon-Chung *et al.*, 1982a). However, combinations between these serotypes exist, as shown by hybrid isolates between both *C. neoformans* varieties (AD hybrids) and interspecies hybrids between *C. neoformans* and *C. gattii* (AB and BD hybrids) (Table 2; Bovers *et al.*, 2006, 2008c). Serotype determination based on cryptococcal capsular antigens was frequently used since it was a reliable method to distinguish *C. neoformans* from *C. gattii* (Ikeda *et al.*, 1982; Kabasawa *et al.*, 1991). Unfortunately, the commercial serotyping test is no longer available and only a few agglutination methods and enzyme immunoassays are currently commercially available, but these lack the possibility to differentiate between the four serotypic groups (Gade *et al.*, 1991; Jaye *et al.*, 1998; Temstet *et al.*, 1992).

DNA fingerprinting methods

The advance of molecular biological tools in cryptococcal research has resulted in a wide variety of techniques that differentiate *C. neoformans* from *C. gattii* more accurately than conventional methods (Table 2). One of the first applied molecular

Previous page, Table 2. [1] Differentiation based on L-canavanine-glycine-bromothymol blue (CGB) medium and creatinine-dextrose-bromothymol blue-thymine (CDBT) medium are given as follows: CGB / CDBT, - = negative and no color change of the CGB medium; + = positive, CGB medium changes to deep blue; w = delayed positive, CGB medium turns pale blue within two weeks; the color change of the CDBT medium are given as M = colour of the medium; C = colour of the colony (Irakanulo *et al.*, 1994; Kwon-Chung *et al.*, 1982); [2] Serotype based on the antigenic composition of the polysaccharide capsule (Bovers *et al.*, 2006, 2008c; Ikeda *et al.*, 1982); [3] Genotypic classification based on AFLP fingerprint typing (Boekhout *et al*, 2001; Bovers *et al.*, 2006; Bovers *et al.*, 2008c; Hagen *et al.*, 2010; Litvintseva *et al.*, 2006); [4] M13 PCR fingerprint typing (Meyer *et al.*, 2009); [5] RFLP analysis based on the *PLB1* gene (Latouche *et al.*, 2003); [6] RFLP analysis based on the *URA5* gene (Meyer *et al.*, 2009); [7] $(GACA)_4$ PCR fingerprinting (Viviani *et al.*, 1997); [8] RFLP analysis based on the *CAP1* and *GEF1* genes (Feng *et al.*, 2008); [9] ITS sequence typing (Imai *et al.*, 2000; Katsu *et al.*, 2004); [10] Intergenic Spacer (IGS1) sequence typing (Diaz *et al.*, 2000). Data that has not been generated is indicated as 'nd'.

techniques was pulsed field gel electrophoresis (PFGE), also referred to as karyotyping (Perfect *et al.*, 1989; Polacheck & Lebens, 1989). With this technique, chromosomes are electrophoretically separated in an agarose gel which resulted in a banding pattern based on chromosomal length differences. Contour-clamped homogeneous field gel electrophoresis (CHEF) and orthogonal field alteration gel electrophoresis (OFAGE) have been applied on cryptococcal isolates (Perfect *et al.*, 1989; Polacheck & Lebens, 1989). These explorative studies showed that clear differences exist between *C. neoformans* and *C. gattii* in terms of number of chromosomes and chromosome lengths, which was subsequently observed for a larger set of cryptococcal isolates (Boekhout *et al.*, 1997).

Restriction enzyme analysis of PCR products (REA-PCR), also called restriction fragment length polymorphism (RFLP) fingerprinting, made use of the ribosomal DNA locus to fingerprint a set of *C. neoformans* and *C. gattii* isolates, as well as other members of the genus *Cryptococcus* (Vilgalys & Hester, 1990). However, *C. neoformans* could not be distinguished from *C. gattii* after enzymatic digestion of the PCR products with four restriction enzymes (Vilgalys & Hester, 1990). This marked the start of a deluge of molecular biological approaches that made use of the PCR amplification technique. The use of DNA fingerprinting was further explored by Meyer and co-workers (1993, 1995) who tested several hybridization probes as single oligonucleotide primer PCR assays. Hybridization probes used for this approach were based on hypervariable and repetitive DNA sequences, such as the M13 phage core sequence, $(GTG)_5$ and $(GACA)_4$. These probes were previously used for conventional DNA fingerprinting for which genomic DNA was enzymatically digested and hybridized with probes that resulted in fingerprint patterns that could discriminate *C. neoformans* and *C. gattii*, but with varying success (Dromer *et al.*, 1994; Polacheck *et al.*, 1992; Spitzer & Spitzer, 1992; Varma & Kwon-Chung, 1992; Varma *et al.*, 1995). The PCR fingerprinting approach as introduced for *Cryptococcus* by Meyer and co-workers (1993), also known as Random Amplification of Polymorphic DNA (RAPD), was a promising method to distinguish not only *C. neoformans* from *C. gattii*, but also the different groups of serotype A and D versus the less polymorphic DNA fingerprints of serotype B and C isolates. Another set of five oligonucleotide primers was tested by Haynes and colleagues (1995) but these were not actively used by the *Cryptococcus* research community.

The first major epidemiological study that took advantage of molecular biological tools was performed on a dataset that included nearly 350 cryptococcal isolates from the USA. For this epidemiological study, another set of primers was used to perform RAPD (Brandt *et al.*, 1995, 1996). A first attempt was made to standardize the procedure of molecular typing, for which a set of over 350 globally collected clinical *C. neoformans* isolates was typed using previously described methods (Meyer *et al.*, 1999). This led to the establishment of four *C. neoformans* genotype designations named VNI and VNII for *C. neoformans* var. *grubii* (serotype A), VNIII for the hybrid between both *C. neoformans* varieties (serotype AD), and VNIV representing *C. neoformans* var. *neoformans* (serotype D). Subsequently, a second study focused on *C. gattii* and revealed the presence of four genotypes named VGI, VGII, VGIII and VGIV (Sorrell *et al.*, 1996). Another research group using the same approach as Meyer *et al.* (1999) and

Sorrell *et al.* (1996) introduced a nomenclature for *C. neoformans* named genotypes VN1 to VN7 and was concordant with other genotyping methods (Table 2; Cogliati *et al.*, 2000; Viviani *et al.*, 1997). Restriction fragment length polymorphism (RFLP) fingerprint methods based on amplification and enzymatic digestion of a particular gene, such as *PLB1* and *URA5*, were found to reveal similar genotypic groupings as were observed with M13 and $(GACA)_4$ DNA fingerprinting and therefore they follow the same genotyping nomenclature (Table 2; Kidd *et al.*, 2004; Latouche *et al.*, 2003; Meyer *et al.*, 2003; Velegraki *et al.*, 2001).

After the introduction of *URA5* RFLP by Casadevall and co-workers (1992), other RFLP methods were introduced but none did reach the status of 'preferred method' (Enache-Angoulvant *et al.*, 2007; Jain *et al.*, 2005; Raimondi *et al.*, 2007). On one hand, this was caused by the fact that the methods did not have sufficiently discriminative power to distinguish certain genotypes, especially within *C. gattii*. On the other hand, this was also due to the fact that M13 DNA fingerprinting and *URA5* RFLP were already well established (Hagen *et al.*, 2007). A promising RFLP method was developed based on the *CAP1* and *GEF1* gene, which was found to discriminate the mating- and genotype combinations, but the banding patterns were difficult to interpret (Table 2; Feng *et al.*, 2008).

Due to its ease and cost-effectiveness, M13 DNA fingerprinting and *URA5* RFLP remained the preferred method to genotype large sets of *Cryptococcus* isolates. However, the Amplified Fragment Length Polymorphism (AFLP) fingerprinting method has been found to be more accurate since it can discern subgenotypes and hybrid genotypes between the two varieties of *C. neoformans* as well as the interspecies hybrids between *C. neoformans* and *C. gattii* (Table 2; Boekhout *et al.*, 2001; Bovers *et al.*, 2006, 2008c; Day *et al.*, 2011; Guinea *et al.*, 2010; Hagen *et al.*, 2010; Litvintseva *et al.*, 2006). Using AFLP fingerprinting, twelve AFLP genotypes can be discerned. Within *C. neoformans* var. *grubii*, three genotypes have been found (AFLP1, AFLP1A and AFLP1B), while *C. neoformans* var. *neoformans* is represented by one major genotype named AFLP2 (Barreto de Oliveira *et al.*, 2004; Boekhout *et al.*, 2001; Guinea *et al.*, 2010; Litvintseva *et al.*, 2006; Mlinarić-Missoni *et al.*, 2011). Hybrids between both *C. neoformans* varieties have been designated as genotype AFLP3. Within *C. gattii* five different haploid genotypes exist that were named AFLP4, AFLP6 and AFLP10 (all contain serotype B isolates) and AFLP5 and AFLP7 (represented by serotype C isolates) (Boekhout *et al.*, 2001; Hagen *et al.*, 2010). The AFLP fingerprinting technique is currently the only fingerprinting method that is able to discriminate between AFLP7 and AFLP10 isolates, and to detect interspecies (aneuploid) hybrids between *C. neoformans* var. *neoformans* AFLP2 × *C. gattii* AFLP4 (AFLP8) and *C. neoformans* var. *grubii* AFLP1 × *C. gattii* AFLP4 (AFLP9) (Table 2; Bovers *et al.*, 2006, 2008c; Hagen *et al.*, 2010).

Due to the existence of multiple nomenclatures for the *C. neoformans* and *C. gattii* genotypes the *Cryptococcus* research community recently adopted the method of Meyer *et al.* (1999) and Sorrell *et al.* (1996) as the preferred way to name genotypes as indicated in Table 2 (Meyer *et al.*, 2009). However, it is frequently used in combination with the genotypic nomenclature that was developed for AFLP genotyping (Table 2).

Sequence-based approaches

The publication of a large study on the phylogeny of basidiomycetous yeasts paved the way for more detailed investigations of specific taxonomic groups (Fell *et al.*, 2000). As a pathogenic basidiomycetous yeast, *C. neoformans* was subsequently investigated using a ribosomal DNA sequence known as the Intergenic Spacer (IGS) region (Diaz *et al.*, 2000). This revealed that significant differences occur in IGS sequences between and within the varieties of *C. neoformans*, at that time including *C. gattii* as a variety. The authors concluded that the *C. neoformans* complex must be split into two species, with *C. neoformans* for serotype A and D isolates and *C. bacillisporus* (= *C. gattii*) for serotype B and C isolates (Diaz *et al.*, 2000). The same research group further explored the use of the IGS region for cryptococcal genotyping and observed that this approach yielded similar results as AFLP fingerprinting (Table 2; Diaz *et al.*, 2005). Another locus that became into fashion as a phylogenetic interesting DNA region for yeast identification and typing was the Internal Transcribed Spacer (ITS), which revealed a similar grouping of *C. neoformans* isolates according to that of PCR fingerprinting (Table 2; Imai *et al.*, 2000; Katsu *et al.*, 2004).

The power of Multi-Locus Sequence Typing (MLST) was for the first time applied by Sugita and colleagues (2001), who combined the sequences of the IGS, ITS, topoisomerase (*TOP1*) and a locus encoding for a capsule associated protein (*CAP59*). Within years, MLST became the standard for typing and epidemiological studies of eukaryotic pathogenic micro-organisms, while this was already a well-established approach for prokaryotic pathogens (Taylor & Fisher, 2003). A start was made to thoroughly revise the taxonomy of the fungal kingdom, with multi-gene phylogenetic analysis as the primary framework (Hibbett *et al.*, 2007; James *et al.*, 2006). Various MLST studies were performed to investigate whether or not the *C. neoformans*/*C. gattii* species complex consists of multiple species (Bovers *et al.*, 2008b, 2009; Ngamskulrungroj *et al.*, 2009; Xu *et al.*, 2009). Additionally, several research groups focused on geographic regions to study cryptococcal populations, especially endemic regions in Africa, Asia, Australia and Vancouver Island were subjected to MLST analysis. (Byrnes *et al.*, 2009a, 2009b, 2010; Carriconde *et al.*, 2011; Chen *et al.*, 2008; Choi *et al.*, 2010; Chowdhary *et al.*, 2011; Fraser *et al.*, 2005; Hiremath *et al.*, 2008; Kidd *et al.*, 2005; Litvintseva *et al.*, 2003, 2007, 2009, 2011; Miglia *et al.*, 2011; Simwami *et al.*, 2011; Xu *et al.*, 2009). These MLST studies revealed that previous assumptions about the epidemiology of *C. neoformans* and *C. gattii* were incorrect. For example, it was observed that *C. gattii* played a major role in cryptococcosis among African HIV-infected individuals, which until then was exclusively conferred to *C. neoformans* (Litvintseva *et al.*, 2005). The opposite was also observed, in China the majority of patients that acquired a *C. neoformans* infection were immunocompetent (Chen *et al.*, 2008)

Due to the fact that the number of MLST studies increased quickly and that each study introduced different MLST schemes (Table 3) a consensus MLST scheme was proposed (Meyer *et al.*, 2009). The two largest MLST studies at that time, on *C. gattii* (Fraser *et al.*, 2005) and *C. neoformans* (Litvintseva *et al.*, 2006), were used to determine which of the loci had the optimal discriminatory power and which combinations of loci

Table 3. Overview of published *Cryptococcus* spp. Multi-Locus Sequence Typing schemes.

Gene	Sugita *et al.*, 2001	Litvintseva *et al.*, 2003	Fraser *et al.*, 2005	Kidd *et al.*, 2005	Litvintseva *et al.*, 2005	Litvintseva *et al.*, 2007	Bovers *et al.*, 2008	Bui *et al.*, 2008	Chen *et al.*, 2008	Hiremath *et al.*, 2008	Bovers *et al.*, 2009	Byrnes *et al.*, 2009a	Byrnes *et al.*, 2009b	Ngamskulrungroj *et al.*, 2009	Xu *et al.*, 2009	Byrnes *et al.*, 2010	Choi *et al.*, 2010	Carriconde *et al.*, 2011	Chowdary *et al.*, 2011	Colom *et al.*, 2011	Litvintseva *et al.*, 2011	Miglia *et al.*, 2011	Simwami *et al.*, 2011
Mitochondrial MLST loci																							
ATP6											×				×								
ATP8															×								
COX1															×								
mtlrRNA										×	×												
NAD2															×								
NAD4															×								
NAD5															×								
Nuclear MLST loci																							
ACT1														×									
BWC1			×									×	×			×							
CAP1				×						×									×				
CAP10			×					×	×			×	×			×				×			
CAP59	×		×		×	×						×				×	×	×	×	×	×		×
CNB1												×				×							
CRG1			×									×				×							
FHB1			×									×				×							
FTR1			×	×						×		×				×			×				
GPD1			×		×	×			×			×	×			×	×	×	×	×	×	×	×
HOG1			×									×	×			×							
IDE1														×					×				
IGS1	×	×	×		×	×	×	×	×		×	×	×			×	×	×	×	×	×	×	×
ITS	×	×					×			×	×												

Table 3. *continued*

LAC1		×	×	×			×		×	×	×	×	×			×	×	×	×	×	×	×
MP88					×	×																
MPD1			×		×	×		×				×	×			×				×		
MS1													×									
PLB1			×		×	×		×	×			×	×	×		×	×	×	×	×	×	×
RPB1		×					×				×											
RPB2							×				×											
SOD1					×	×		×	×								×	×		×	×	×
SXI1α			×									×	×			×						
SXI2a			×									×	×			×						
TEF1			×		×	×	×		×		×	×	×			×				×	×	
TOP1	×				×	×		×														
TOR1			×									×	×			×						
URA5				×										×			×	×	×	×	×	×
URE1			×		×	×			×													

Gene names refers to those used for the genome sequenced reference strains (D'Souza *et al.*, 2011; Loftus *et al.*, 2005). Shaded rows indicate the seven nuclear loci selected for the consensus MLST scheme (Meyer *et al.*, 2009). The MLST study by Fraser *et al.* (2005) also used the sequences of the genes *BWC2, CAC1, CBP1, CCP1, CNA1, CPK1, GPA1, ICL1, PAK1, RAS1, STE7, TRR1* and *TSA1* for a subset of the investigated *C. gattii* strains.

were suitable to obtain the best level of diversity with the lowest number of loci to be studied. This showed that a seven-loci MLST scheme had an optimal discriminatory power and that the addition of more loci did not largely influence the genetic diversity observed. Therefore a consensus MLST was proposed that contains the seven unlinked nuclear loci *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5* (Meyer *et al.*, 2009).

However, it will not take long before MLST will be overtaken by whole genome sequencing (WGS) as the favorable approach for sequence analysis. During the past years WGS has gone through turbulent developments that have speed up the generation of reliable sequence data and its analysis and this has already resulted in more than thousand sequenced yeast genomes (Dujon *et al.*, 2010). Among them are the genomes of reference strains representing *C. neoformans* var. *grubii* (H99), var. *neoformans* (JEC21) and *C. gattii* genotype AFLP4 and AFLP6 (WM276 and A1M-R265, respectively) (D'Souza *et al.*, 2011; Loftus *et al.*, 2005). The availability of genomic data from various genotypic cryptococcal strains has not only resulted in new sophisticated methodologies, such as micro-array analysis, but also in new epidemiological typing methods that could be developed using whole genome databases. One of these typing methods is based on simple tandem repeat sequences, or microsatellites, which were

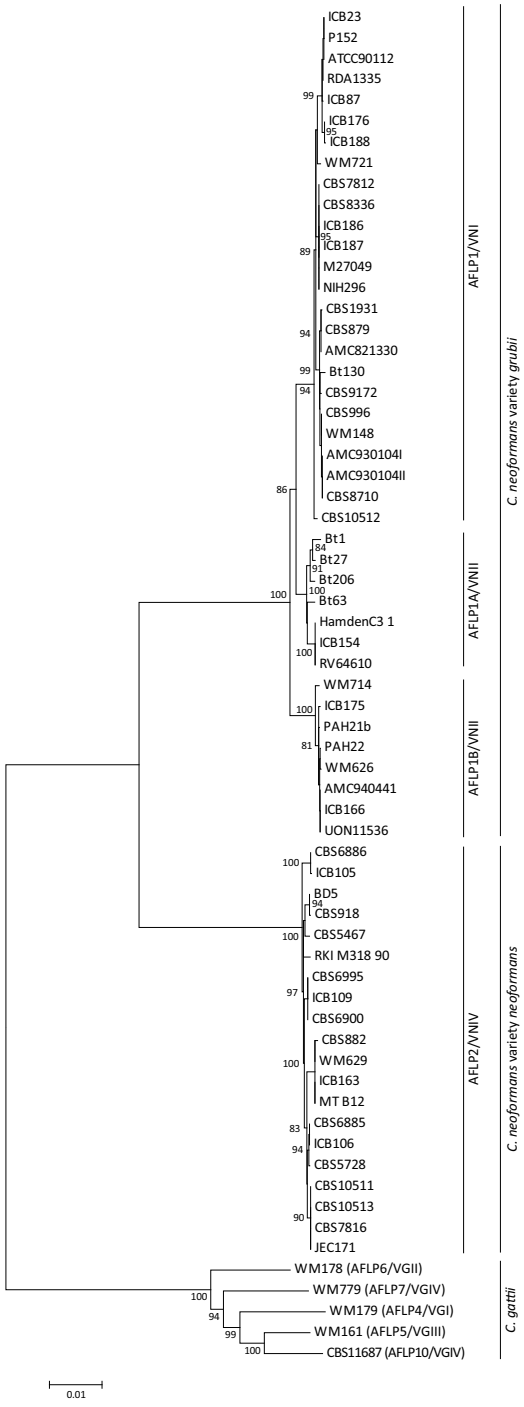

Figure 2. Bootstrap Maximum Likelihood phylogenetic analysis of *Cryptococcus neoformans* strains based on eleven unlinked nuclear loci (6650 nt) according to the consensus MLST scheme and AFTOL loci (Bovers *et al.*, 2008b; F. Hagen and T. Boekhout, unpublished data). Five *C. gattii* reference strains were used as outgroup. Observed *C. neoformans* groups cluster according to their AFLP and PCR fingerprinting pattern, with AFLP1/VNI, AFLP1A/VNII and AFLP1B/VNII for *C. neoformans* var. *grubii* and AFLP2/VNIV for *C. neoformans* var. *neoformans* isolates (Boekhout *et al.*, 2001). Numbers next to the branches shows highly supportive bootstrap values (≥75).

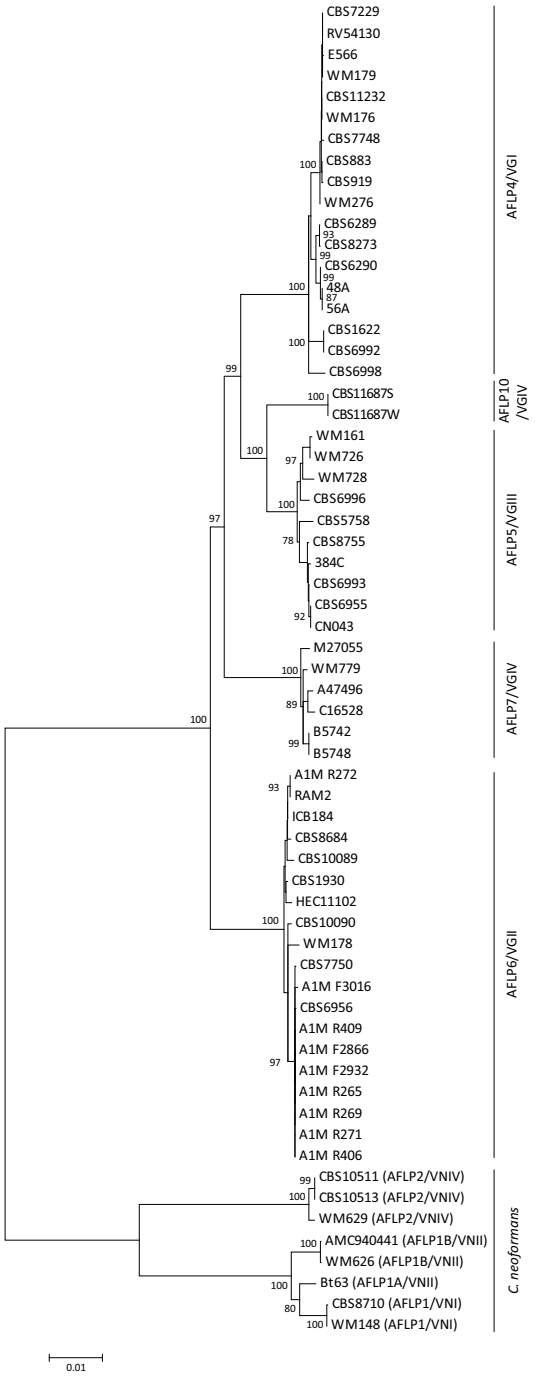

Figure 3. Bootstrap Maximum Likelihood phylogenetic analysis of *Cryptococcus gattii* strains based on eleven unlinked nuclear loci (6650 nt) according to the consensus MLST scheme and AFTOL loci (Bovers *et al.*, 2008b; F. Hagen and T. Boekhout, unpublished data). Eight *C. neoformans* reference strains were used as outgroup. Observed *C. gattii* groups cluster according to their AFLP and PCR fingerprinting pattern, with AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV (Boekhout *et al.*, 2001; Hagen *et al.*, 2010). Numbers next to the branches shows highly supportive bootstrap values (≥75).

found to be abundantly present in the genome of *C. neoformans* var. *grubii* when compared to other available fungal genomes (Karaoglu *et al*., 2005). Currently, multiple Multi-Locus Microsatellite Typing (MLMT) schemes have been developed for *C. neoformans* and *C. gattii*, and it has been shown that this typing method is portable and cost effective when compared to MLST (Hanafy *et al.*, 2008; Illnait-Zaragozí *et al.*, 2010, 2011; Karaoglu *et al.*, 2008).

Taxonomic impact of molecular typing

Molecular typing has revealed the existence of discrepancies in the taxonomy of the fungal kingdom (Hibbett *et al*., 2007; James *et al.*, 2006). For *C. neoformans* and *C. gattii*, recent molecular studies did not reveal unexpected changes into the already established taxonomy, but it is not a surprise that molecular typing has fueled the debate to further differentiate the *C. neoformans*/*C. gattii* species complex into different species instead to use the genotypic classification based on AFLP fingerprinting, PCR fingerprinting and MLST (Bovers *et al.*, 2008a, 2008b; Ngamskulrungroj *et al.*, 2009). During the past years, the species delineation of the *C. neoformans*/*C. gattii* species complex has served as an example of ongoing debate among fungal taxonomists, researchers and clinicians (Kwon-Chung & Varma, 2006). MLST has repeatedly shown that *C. neoformans* and *C. gattii* contain monophyletic clusters, which is consistent with observations based on other genotypic and phenotypic methods (Figure 2 and 3; Table 2). Within *C. neoformans* var. *grubii*, three distinct fingerprint genotypes have been observed (AFLP1, AFLP1A and AFLP1B) which is in agreement with MLST genotyping (Figure 2; Table 2; Boekhout *et al.*, 2001; Bovers *et al.*, 2008; Choi *et al.*, 2010; Litvintseva *et al.*, 2006; Ngamskulrungroj *et al.*, 2009). Isolates that belong to var. *neoformans* consistently form a monophyletic cluster based on fingerprinting and sequence-based methods (Figure 2; Boekhout *et al.*, 2001; Bovers *et al.*, 2006; Ngamskulrungroj *et al.*, 2009). For *C. gattii*, it has already been suggested that the monophyletic clusters within this species, namely the five genotypes AFLP4, AFLP5, AFLP6, AFLP7 and AFLP10, should be raised to the variety level (Ngamskulrungroj *et al.*, 2009). Similar to the phylogenetic observations of *C. neoformans*, monophyletic clusters are consistently observed within *C. gattii*, as shown in Figure 3, and these may be interpreted as species (Boekhout *et al.*, 2001; Bovers *et al.*, 2008; Fraser *et al.*, 2005; Ngamskulrungroj *et al.*, 2009).

Based on an overwhelming number of molecular studies, taxonomists and researchers seems to be convinced of the necessity to further differentiate the *C. neoformans*/*C. gattii* complex into seven species (Table 4). However, two hurdles must still be taken by taxonomists. First of all, appropriate species names needs to be chosen, not only based on historical descriptions of *C. neoformans* and *C. gattii* synonyms, but also based on suggestions from research and clinical communities. Secondly, clinicians will not directly embrace the introduction of novel cryptococcal species that all show indistinguishable clinical signs, and that cannot be differentiated by microscopy or other easy-to-use microbiological methods. It is obvious that novel species will be introduced based on the monophyletic clusters within the *C. neoformans*/*C. gattii*

Table 4. Proposed classification of the *C. neoformans*/*C. gattii* species complex

Current name	Genotype	Serotype	Proposed name	Type-strain	Reference
C. neoformans var. *grubii*	AFLP1	A	*C. neoformans*	CBS8710	Franzot *et al.*, 1999
C. neoformans var. *neoformans*	AFLP2	D	*C. deneoformans**	CBS882	Harrison, 1928
C. gattii	AFLP4	B	*C. gattii*	CBS6289	Curtis, 1896
C. gattii	AFLP5	C	*C. bacillisporus*	CBS6955	Kwon-Chung *et al.*, 1982
C. gattii	AFLP6	B	Not yet proposed	CBS10082	Bovers *et al.*, 2008
C. gattii	AFLP7	C	Not yet proposed	CBS10101	Bovers *et al.*, 2008
C. gattii	AFLP10	B	Not yet proposed	CBS11687	Hagen *et al.*, 2010a
C. neoformans intervariety hybrid	AFLP3	AD	*C. neoformans* × *C. deneoformans*	CBS132	Sanfelice, 1895
C. neoformans var. *neoformans* × *C. gattii*	AFLP8	BD	*C. deneoformans* × *C. gattii*	CBS10488	Bovers *et al.*, 2006
C. neoformans var. *grubii* × *C. gattii*	AFLP9	AB	*C. neoformans* × *C. gattii*	CBS10496	Bovers *et al.*, 2008c

* The taxonomic valid name is *C. nasalis* (CBS882) but this is not supported by the cryptococcal research community, on the contrary to the proposed name *C. deneoformans* (T. Boekhout and J. Heitman, personal communication).

species complex, but this will not go without any resistance from the various disciplines in the cryptococcal research field. Hence, such a decision should be based on various phenotypic and molecular epidemiological data and the studies described in this thesis will contribute to this.

Outline of this thesis

Until three decades ago the basidiomycetous yeast species *C. neoformans* and *C. gattii* were rarely encountered as pathogens. This changed during the 1980s with the sudden onset of the HIV-pandemic, that coincided with a sharp increase of cryptococcal infections, and due to the unprecedented *C. gattii* outbreak that emerged in 1999 in British Columbia, Canada. Until recently, it was believed that *C. neoformans* infections occur mainly in immunocompromised patients while *C. gattii* had a predilection to infect apparently immunocompetent individuals. With the advent of molecular techniques it was observed that the epidemiology of *C. neoformans* and *C. gattii* is more complex than was assumed. In this thesis *C. neoformans* and *C. gattii* populations are genotypically studied using conventional and novel molecular epidemiological approaches in combination with the characterization of virulence related traits.

This thesis consists of three sections: Section I contains the Chapters 2, 3 and 4 that focus on the epidemiology of *C. neoformans* in Croatia, the Netherlands and Spain. Section II includes Chapters 5, 6 and 7 that focus on the epidemiology of *C. gattii*. Section III comprises the Chapters 8, 9, 10 and 11 that are all dedicated to the epidemiological and clinical aspects of the expanding *C. gattii* outbreaks in North America.

Chapter 2 describes the epidemiology of cryptococcosis in the Netherlands during the period 1977-2007. The Dutch *Cryptococcus* population is investigated using AFLP fingerprinting, mating- and serotyping by PCR, and microsatellite typing specific for isolates with a genotype AFLP1 or AFLP2 background. Additionally, antifungal susceptibility was performed using seven antifungal compounds. A comparison between genotypic data, *in vitro* antifungal susceptibility profiles and patient categories are given. The epidemiological studies applied on clinical *C. neoformans* isolates from Spain and Croatia are described in Chapter 3 and 4, respectively, with a similar approach as that for the Dutch *Cryptococcus* study.

Chapter 5 describes the occurrence and genotypic diversity of clinical, veterinary and environmental *C. gattii* isolates from Europe. MLST was used to investigate the genotypic structure of the European *C. gattii* isolates and a comparison was made with a set of globally collected isolates. This revealed that European *C. gattii* infections are mainly travel-related and that *C. gattii* is endemic in Mediterranean Europe. The presence of *C. gattii* in Mediterranean Europe is the topic of Chapter 6 that describes the presence of *C. gattii* genotype AFLP4 in the environment of Spain and that clinical, veterinary and environmental isolates share a unique MLST profile. The *in vitro* antifungal susceptibility profiles for a collection of 350 globally collected *C. gattii* isolates is described in Chapter 7. This study shows that *C. gattii* genotype AFLP6

isolates are significantly less susceptible to antifungals than genotype AFLP4 isolates.

Chapter 8 focuses on the global population structure of *C. gattii* genotype AFLP6 that is involved in multiple outbreaks. Using four independent molecular epidemiological approaches and coalescence gene genealogy it is shown that the geographic origin of these outbreaks is in South America. Next to this, macrophage and mice pathogenicity assays were used to show that hypervirulence is present among the oldest lineages. The virulent phenotype of isolates from different *C. gattii* genotypes is described in Chapter 9. The application of macrophage and mice virulence assays revealed enhanced virulence for *C. gattii* genotype AFLP6 isolates. Subsequent microarray based investigations showed that this is due to genetic and phenotypic differences in the mitochondrial architecture. Finally, in Chapter 10 and 11 two European case reports of the *C. gattii* related outbreak in North America are presented that highlights the importance of molecular epidemiology in the investigation of cryptococcal infections.

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Chapter 2

Extensive genetic diversity within the Dutch clinical *Cryptococcus neoformans* population

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Submitted.

Summary

A set of 300 Dutch *Cryptococcus neoformans* isolates, obtained from 237 patients during the period 1977 to 2007, was investigated to determine the mating- and serotype, AFLP genotype and susceptibility for seven antifungal compounds. Almost half the studied cases of cryptococcosis occurred in HIV-infected patients, followed by a patient group with other underlying diseases and immunocompetent individuals. The majority of the isolates were mating-type α and serotype A, followed by serotype D isolates and other minor categories. The most frequently observed genotype was AFLP1, distantly followed by AFLP2 and AFLP3. Microsatellite typing revealed a high genetic diversity among serotype A isolates, but this was found to be more limited within the set of serotype D isolates. One patient was found to be infected by multiple AFLP genotypes. Fluconazole and 5-flucytosine had the highest geometric mean minimal inhibitory (MIC) concentration of 2.865 and 3.505 μg/ml, respectively, while amphotericin B (0.242 μg/ml), itraconazole (0.082 μg/ml), voriconazole (0.071 μg/ml), posaconazole (0.058 μg/ml) and isavuconazole (0.033 μg/ml) had much lower geometric mean MIC values. One isolate was found to be resistant against 5-flucytosine, and decreased susceptibility (≥16 μg/ml) for 5-flucytosine and fluconazole was found for nine and ten *C. neoformans* isolates, respectively.

Introduction

The encapsulated basidiomycetous yeast *Cryptococcus neoformans*, the causative agent of cryptococcosis, can cause life threatening infections such as severe lung infections and meningoencephalitis. This fungal disease was rarely seen until the early 1980s but this changed due to the HIV pandemic (Bovers *et al.*, 2008a; Warkentien & Crum-Cianflone, 2010; Van Elden *et al.*, 2000). Annually nearly one million HIV-infected subjects develop cryptococcosis, mainly in sub-Saharan Africa where the majority of patients die due to ineffective or lack of treatment options (Park *et al.*, 2009; Warkentien & Crum-Cianflone, 2010). Besides a high incidence of cryptococcosis among HIV-infected patients, individuals with other underlying diseases might be at risk due to immune disorders or immunosuppressive therapy (e.g. organ transplant recipients, and individuals receiving anti-cancer therapy) (Bovers *et al.*, 2008a; Park *et al.*, 2009; Warkentien & Crum-Cianflone, 2010).

The genus *Cryptococcus* contains over 100 species according to the current taxonomic classification (Fonseca *et al.*, 2011). However, only *C. neoformans* and *C. gattii* are a frequent cause of cryptococcal disease in humans and animals. *Cryptococcus gattii* has been recognized as a primary pathogen and *C. neoformans* is worldwide known as an opportunist. The latter is currently divided into two varieties, *C. neoformans* var. *grubii* (serotype A) and var. *neoformans* (serotype D) (Bovers *et al.*, 2008a). The third variety, *C. neoformans* var. *gattii* (serotype B and C), was raised to a separate species named *C. gattii* (Bovers *et al.*, 2008a; Kwon-Chung *et al.*, 2002).

PCR fingerprinting, Restriction Fragment Length Polymorphism analysis of the *PLB1* and *URA5* locus, Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis and several Multi-Locus Sequence Typing (MLST) studies divided *C. neoformans* and *C. gattii* into ten distinct genotypes (Boekhout *et al.*, 2001; Bovers *et al.*, 2008b; Kidd *et al.*, 2004; Meyer *et al.*, 2009; Ngamskulrungroj *et al.*, 2009). Within *C. neoformans* var. *grubii*, three genotypes can be discerned named AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII, var. *neoformans* (AFLP2/VNIII) and the intervariety serotype AD hybrid (AFLP3/VNIV). *Cryptococcus gattii* can be divided in five genotypes: AFLP4/VGI, AFLP6/VGII and AFLP10/VGIV with serotype B isolates, while AFLP5/VGIII and AFLP7/VGIV are represented by serotype C isolates (Hagen *et al.*, 2010a; Meyer *et al.*, 2009). Currently, two interspecies hybrids have been described, *C. neoformans* var. *neoformans* AFLP2/VNIII × *C. gattii* AFLP4/VGI is known as genotype AFLP8 and *C. neoformans* var. *grubii* AFLP1/VNI × *C. gattii* AFLP4/VGI has been designated genotype AFLP9 (Bovers *et al.*, 2006, 2008c).

A retrospective survey among HIV-infected patients in the Netherlands identified 268 patients with cryptococcosis during the period 1986-1999 (Van Elden *et al.*, 2000). A major increase in the incidence of the disease was observed since 1987, which correlated with the increasing number of HIV/AIDS patients. The introduction of highly active antiretroviral therapy (HAART) in 1996 resulted in a significant decrease of cryptococcosis patients in the Netherlands (Van Elden *et al.*, 2000). A clinical epidemiological analysis was made on the patient characteristics, but a genotypic analysis of the involved *C. neoformans* isolates has not been performed.

In this study we investigated Dutch *C. neoformans* isolates that were collected during a 30-year period (1977-2007) to investigate the genetic diversity. AFLP fingerprinting, microsatellite typing and mating- and serotype analysis using PCRs were performed to assess the genetic diversity among Dutch clinical *Cryptococcus* strains. Antifungal susceptibility profiles were determined for the antifungal compounds amphotericin B, 5-flucytosine, fluconazole, itraconazole and the three novel triazoles posaconazole, voriconazole and isavuconazole.

Material and Methods

Patient related data
Clinically relevant information was extracted from the database of the Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center, Amsterdam, The Netherlands) and included the source and date of *Cryptococcus* isolation, date of birth, gender, and place of residence of the patient. Patient data was requested from the contributing clinicians or hospitals in the case that no or limited data was present. Relapses of cryptococcal infections were defined by a period between two positive cultures of more than ninety days. Four patient categories were determined based on the predisposing factors that may have influenced the onset of the *Cryptococcus* infection, namely HIV/AIDS related immunodeficiency, non-HIV/AIDS related immunodeficiency (e.g. immunosuppressive therapy, organ transplantation), immunocompetent individuals, and a patient category for which the presence or absence of predisposing factors were unknown.

Cryptococcal isolates and media
Cryptococcal isolates were revived from the -80°C collection of the Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center, Amsterdam, The Netherlands) or were requested from participating Dutch hospitals. Isolates were cultured on YPGA medium containing 1% yeast-extract, 1% peptone, 2% D-glucose and 2% agar, and incubated at 25°C for 2 days.

Genomic DNA extraction
Isolates were subcultured for 2 days at 30°C on YPGA media supplemented with 0.5 M NaCl to prevent the formation of polysaccharide capsules. Genomic DNA was extracted as described previously (Hagen *et al.*, 2010a). The purity and quantity of the genomic DNA solution was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) and final work solutions with a concentration of 100 ng/µl were prepared.

Mating- and serotyping by PCR and AFLP genotyping
Mating- and serotype determination of the isolates was performed as described previously (Barreto de Oliveira *et al.*, 2004). Reference strains 125.91 (= CBS10512; **a**A; AFLP1), H99 (= CBS8710; αA; AFLP1), JEC20 (= CBS10511; **a**D; AFLP2) and JEC21

(= CBS10513; αD; AFLP2) were included as controls. Isolates that failed to yield an amplicon were subsequently subjected to a set of two PCRs to amplify the *C. gattii* mating-type *STE12***a** or *STE12α* allele (Bovers *et al.*, 2006). For these PCRs, the reference strains CBS1930 (**a**B; AFLP6) and WM276 (αB; AFLP4) were included as controls.

The genotype was determined using AFLP fingerprint analysis as described previously by Boekhout and co-workers (2001). Reference strains for each of the genotypes were included according to Hagen and colleagues (2010a).

Microsatellite amplification and analysis

The genetic relatedness of *C. neoformans* var. *grubii* (serotype A) isolates was further investigated using nine short tandem repeat markers (STRs) as described recently (Illnait-Zaragozí *et al.*, 2010). Fragment analysis on a MegaBACE™ 500 capillary electrophoresis platform (GE Healthcare Life Sciences, Piscattaway, PA) was carried out as described by Illnait-Zaragozí and colleagues (2010). Bionumerics v6.1 (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate a minimum spanning tree. A multistate categorical similarity coefficient was used, and microsatellite complexes (MCs) were defined by a minimum size of two types that contain at least five isolates and with a maximum neighbor distance of a two loci difference among the nine loci studied (Illnait-Zaragozí *et al.*, 2010).

To characterize the genetic structure of *C. neoformans* var. *neoformans* (serotype D) isolates, a microsatellite panel was developed based on the genome sequence of reference strain JEC21 using the Tandem Repeats Finder software package (Benson, 1999). This panel consists of two dinucleotide repeat loci, four trinucleotide repeat loci

Table 1. Primer sequences for microsatellite typing of *C. neoformans* var. *neoformans*.

Primer	Primer sequence	Target
CND2-1 Fwd	AATCCTGAGACGGTTTGTGG [FAM dye at 5′ side of the primer]	CND2A
CND2-1 Rvd	GAACATTGTGCCCGACTTTT	
CND2-4 Fwd	ATCGAAGGTCTCGTCGTCTG [TET dye at 5′ side of the primer]	CND2B
CND2-4 Rvd	GAATGCTCTGGATGGAAGGA	
CND3-1 Fwd	TGCTGAGCTTGTTGAGGTTG [FAM dye at 5′ side of the primer]	CND3A
CND3-1 Rvd	GAATGGCGCACTAATCTACCC	
CND3-2 Fwd	AGGCAAGACTGACGTTGAGC [HEX dye at 5′ side of the primer]	CND3B
CND3-2 Rvd	GCAACTTCCTCCCCTCTTTCC	
CND3-3 Fwd	GTTGGTCTGTCTCCCTCGAT	CND3C
CND3-3 Rvd	TTTCAAAGATGCTTGATGAGGA [TET dye at 5′ side of the primer]	
CND3-4 Fwd	GTCGGTAAGGTTTTGGGTTGA	CND3D
CND3-4 Rvd	AGCACCAAAAGATGGGTACAA [HEX dye at 5′ side of the primer]	
CND4-3 Fwd	GCGCCGCTCTTAGAATGAAGA	CND4A
CND4-3 Rvd	TCTCCGTCTGTCGTCTTTTG [TET dye at 5′ side of the primer]	

and one tetranucleotide repeat locus (Table 1). Either the forward or reversed primer was labeled with a fluorescent dye (FAM, HEX or TET). Amplification conditions, processing of amplicons and data analysis were identical to those used for the analysis of the *C. neoformans* var. *grubii* microsatellite panel (CNA).

Antifungal susceptibility testing

The minimal inhibitory concentrations (MIC) of amphotericin B (Bristol Myers Squibb, Woerden, The Netherlands), 5-flucytosine (Valeant Pharmaceuticals, Zoetermeer, Netherlands), fluconazole and voriconazole (Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (Janssen Cilag, Tilburg, The Netherlands), posaconazole (Schering-Plough Corp., Kenilworth, NJ), and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) were determined using a broth microdilution method, in accordance with the guidelines of the CLSI document M27-A3 (National Committee for Clinical Laboratory Standards, 2008). The procedure was followed as described by Hagen and colleagues (2010a).

Statistical analysis

The MIC_{50} and MIC_{90} were determined by ordering the MIC values for each antifungal in ascending arrays and selecting the median and 90th quantile, respectively, of the MIC distribution. Geometric mean MICs were calculated using Office Excel 2007 (Microsoft, Redmond, WA), for which purpose values "<X" were set equal to "0.5 X" and ">Y" were set equal to "2Y". A two-tailed Mann-Whitney-Wilcoxon test was performed to compare the MIC values between different groups, and a *P*-value of ≤0.05 was regarded as statistically significant. Significant correlations between *Cryptococcus* characteristics versus any of the other values were calculated using a Chi square test. Statistical analyses were performed using the SAS software package (SAS, Cary, NC). Discriminatory power of the microsatellite typing experiments was calculated using the software package Haplotype Analysis v1.05 (Eliades & Eliades, 2009).

Results

Cryptococcal isolates, patients and patient categories

Three-hundred of the 328 isolates from the frozen stock cultures were viable and included in this epidemiological study. Detailed data of the total number of *Cryptococcus* isolates and the number of patients included, as well as number of isolates per defined patient category, are listed in Table 2. The isolates were isolated from cerebrospinal fluid (CSF) (*n*=201; 67.0%), blood (*n*=45; 15.0%), lung (*n*=28; 9.3%), bone (*n*=5; 1.7%), spleen, urine (each *n*=2; 0.67%), pus, neck gland (each *n*=1; 0.33%) and unknown sources (*n*=15; 5.0%).

The 300 isolates came from 237 patients of whom 45 were female (19.0%), 188 were males (79.3%) and of four patients the gender remained unknown (1.7%). The ratio male to female patients was 4.2 to 1 with an overall age distribution of 14 to 83 years and male and female age distributions of 17 to 83 and 14 to 78 years, respectively.

Table 2. Number of included *Cryptococcus* isolates, cryptococcosis patients and age distribution overall and per gender

		Total	HIV/AIDS	Other predisposing factors	Immuno-competent	Unknown immune status
***Cryptococcus* isolates**	n_{ISOLATES} (%) [1]	300 (100)	137 (45.7)	68 (22.7)	24 (8.0)	71 (23.7)
	$n_{\text{CSF CULTURE}}$ (%) [2]	201 (67.0)	105 (76.6)	37 (54.4)	13 (54.2)	46 (64.8)
	$n_{\text{BLOOD CULTURE}}$ (%) [2]	45 (15.0)	20 (14.6)	7 (10.3)	4 (16.7)	13 (18.3)
	$n_{\text{OTHER SOURCE}}$ (%) [2]	54 (18.0)	12 (8.8)	24 (35.3)	7 (29.2)	12 (16.9)
All patients	n_{PATIENTS} (%) [1]	237 (100)	115 (48.5) [3,4]	50 (21.1) [3]	18 (7.6)	54 (22.8) [5]
	Male : Female ratio	4.2 : 1	8.4 : 1	1.8 : 1	3.5 : 1	3.5 : 1
	Age distribution (year)	14 - 83	20 – 63 [3]	17 - 78 [3]	15 - 83	14 -69
	Average age (year)	43.2	38.2 [3]	53.9 [3]	50.8	41.2
	Median age (year)	41	37 [3]	60 [3]	48	38
Male patients	$n_{\text{MALE PATIENTS}}$ (%) [1]	194 (100)	101 (53.7) [4]	31 (16.5)	14 (7.4)	42 (22.3) [5]
	Age distribution (year)	17 - 83	20 – 63	17 – 75	27 - 83	19 – 69
	Average age (year)	42.7	39.0	52.7	53.1	40.6
	Median age (year)	41	38	59	48	35
Female patients	$n_{\text{FEMALE PATIENTS}}$ (%) [1]	45 (100)	12 (26.7)	17 (37.8)	4 (8.9)	12 (26.7)
	Age distribution (year)	14 - 78	23 – 43	30 - 78	15 - 73	14 - 69
	Average age (year)	45.4	31.0	53.8	43.0	42.9
	Median age (year)	42	32.8	60	42	49

The number of included *C. neoformans* isolates is listed for the categories 'HIV/AIDS patients', 'Patients with other predisposing factors', 'Immunocompetent patients', and 'Patients with an unknown immune status'. Data are divided in four sections: providing the source of the cultured isolates, patient related data, and patient related data for male and female patients. [1] Numbers and percentages indicated in these patient categories are the cumulative number and percentage indicated in the column 'Total'. [2] Numbers and percentages of isolates per source (CSF, blood or other source) are the cumulative number and percentage of isolates applied to that patient category. [3] Including two patients without known gender. [4] Including one male patient without known age. [5] Including three male patients without known age.

The average age at which the 237 patients developed cryptococcosis was 43.2 years with a median of 41 years. Almost half the studied cases of cryptococcosis occurred in HIV-infected patients (*n*=115; 48.5%), followed by the patient group with other underlying diseases (*n*=50; 21.1%), immunocompetent individuals (*n*=18; 7.6%), and 54 (22.8%) of the episodes occurred in patients for which no clinical data were available. Two or more isolates were available from 42 patients (17.7%), and two of these patients had a relapse of cryptococcosis.

Mating-, sero- and AFLP genotyping

Detailed data on the mating-type, serotype and AFLP genotype distribution among investigated *Cryptococcus* spp. isolates per patient category are listed in Table 3. PCR based mating- and serotyping revealed that the majority of the 300 isolates were αA (*n*=219; 73.0%). Thirty-eight isolates (12.7%) were serotype D of which 32 were mating-type α and six mating-type **a**. The remaining isolates belonged to minor mating- and serotype categories as listed in Table 3.

The mating- and serotype of nine isolates (3.0%) could not be determined and these were subsequently subjected to a *C. gattii* specific PCR. This resulted in two

Table 3. Genetic background of *Cryptococcus* spp. isolates per patient category

		Total [1]	HIV/AIDS [1]	Other predisposing factors [1]	Immuno-competent [1]	Unknown immune status [1]
PCR based mating- and serotyping	αA (%)	219 (73.0)	95 (69.3)	47 (69.1)	20 (83.3)	57 (80.3)
	aD (%)	6 (2.0)	3 (2.2)	2 (2.9)	-	1 (1.4)
	αD (%)	32 (10.7)	9 (6.6)	13 (19.1)	1 (4.2)	9 (12.7)
	αA**a**A (%)	21 (7.0)	14 (10.2)	2 (2.9)	2 (8.3)	3 (4.2)
	αAαD (%)	1 (0.3)	1 (0.7)	-	-	-
	αA**a**D (%)	6 (2.0)	5 (3.6)	1 (1.5)	-	-
	αD**a**A (%)	4 (1.3)	4 (2.9)	-	-	-
	C. gattii (α) (%)	2 (0.7)	1 (0.7)	1 (1.5)	-	-
	Unknown (%)	9 (3.0)	5 (3.6)	2 (2.9)	1 (4.2)	1 (1.4)
AFLP genotyping	AFLP1 (%)	245 (82.0)	111 (81.0)[4]	51 (75.0)[3]	22 (91.7)[2]	62 (87.3)
	AFLP2 (%)	36 (11.7)	13 (9.5)	13 (19.1)	1 (3.8)	8 (11.3)
	AFLP3 (%)	14 (4.7)	12 (8.8)	1 (1.5)	1 (3.8)	-
	AFLP4 (%)	1 (0.3)	1 (0.7)	-	-	-
	AFLP6 (%)	1 (0.3)	-	1 (1.5)	-	-
	AFLP8 (%)	3 (1.0)	-	2 (2.9)	-	1 (1.4)

[1] Percentages listed in this column are cumulative for this column per section 'PCR based mating- and serotyping' and 'AFLP genotyping'; [2] Includes one isolate with genotype AFLP1B; [3] Includes two isolates with genotype AFLP1B; [4] Includes four isolates with genotype AFLP1B.

(0.7%) isolates that were *C. gattii* mating-type α, while the remaining seven (2.3%) isolates remained untypeable.

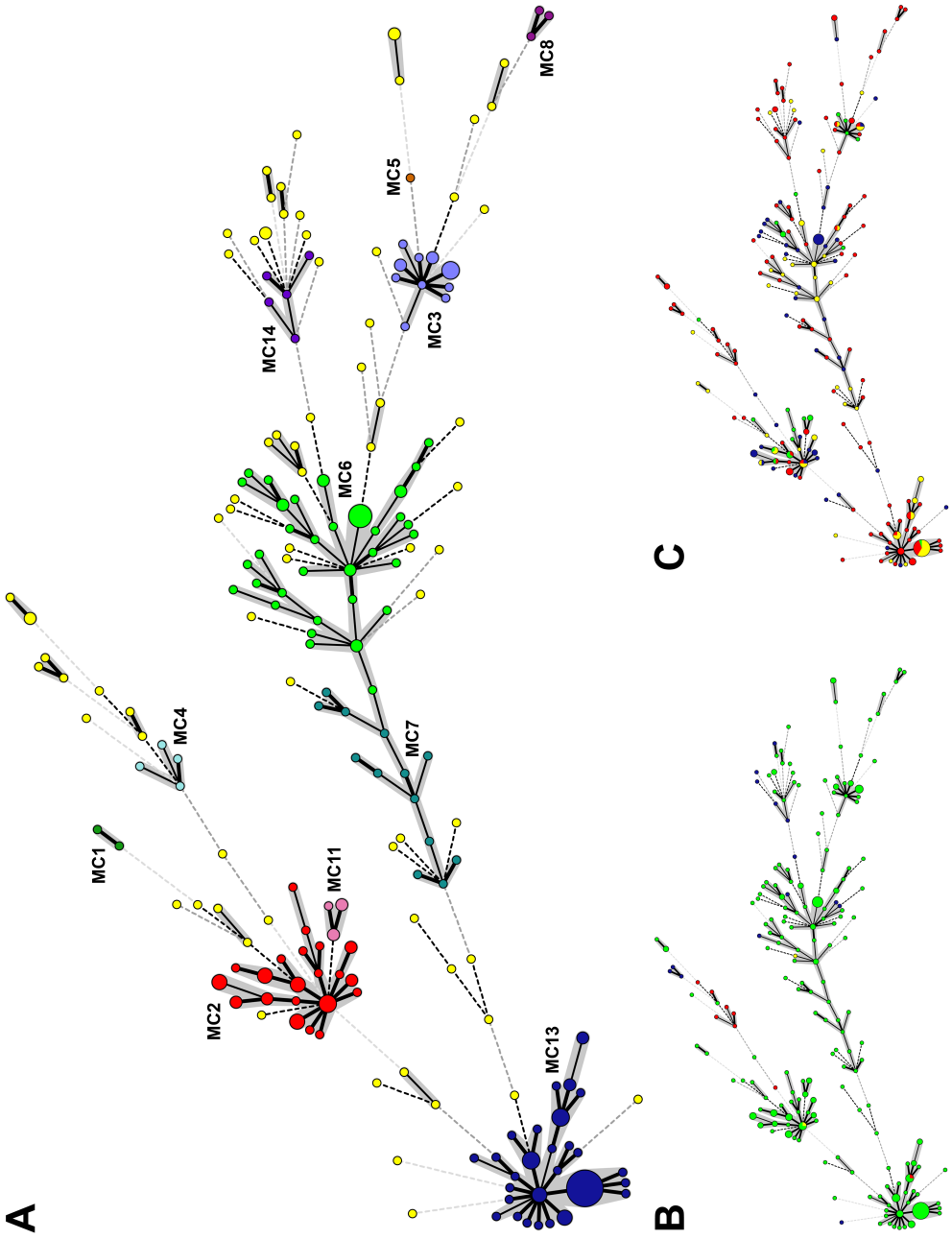
Amplified Fragment Length Polymorphism (AFLP) fingerprinting divided the isolates into six AFLP groups. The majority of isolates (*n*=245; 81.7%) clustered together with reference strains for genotype AFLP1 which represents *C. neoformans* var. *grubii* (serotype A). Thirty-six isolates clustered together with the reference strain for genotype AFLP2 (*n*=36; 12.0%) representing *C. neoformans* var. *neoformans* (serotype D), and fourteen clustered together in genotype AFLP3 (*n*=14; 4.7%) that represents the serotype AD hybrids. The remaining isolates belonged to minor AFLP genotypes, as listed in Table 3.

Some discrepancies between the conventional mating- and serotyping method versus AFLP fingerprinting were observed. Four isolates (1.2%) were determined by mating- and serotyping PCRs as αA or **a**D, but AFLP genotyping revealed that they were actually hybrid serotype AD isolates (AFLP3) that lacks either the serotype A or D genetic background. Three isolates that were determined by mating-serotyping PCR as **a**D, were identified by AFLP genotyping as interspecies hybrids between *C. neoformans* var. *neoformans* and *C. gattii* that were assigned AFLP8 (Bovers *et al.*, 2006). A significant correlation between the genotypic AFLP1 and AFLP2 groups versus the immune status of the patients was observed (*P*=0.029).

Microsatellite genotyping of serotype A isolates

Based on serotype and AFLP fingerprint analysis, a set of 259 *C. neoformans* isolates was further investigated using a serotype A specific microsatellite panel. This included 245 serotype A isolates belonging to genotype AFLP1 and 14 serotype AD hybrid isolates belonging to genotype AFLP3 (Table 3).

When all nine microsatellite CNA markers were combined, 196 different microsatellite genotypes and eleven MCs could be distinguished among 259 isolates (Figure 1). Using the Simpsons diversity index (*D*), the discriminatory power for the complete set of nine microsatellite markers was 0.994, and that of the three separate panels 0.929, 0.953 and 0.985 for CNA2, CNA3 and CNA4, respectively. Among the nine studied microsatellite loci, CNA4A had the highest genotypic diversity with 59 types (*D*=0.967), while locus CNA3B had the lowest genotypic diversity with nine types (*D*=0.649). However, locus CNA3C showed the lowest resolution with a *D*-value of 0.578. Microsatellite loci CNA3A and CNA4A showed a relatively high degree of negative results for 35% and 6% of the investigated isolates, whereas the other microsatellite markers showed only occasionally absence of a microsatellite locus. The assignment of microsatellite clusters (MCs) remained intact when microsatellite loci CNA3A and CNA4A were discarded from the analysis (data not shown). A step-by-step removal of the most discriminatory microsatellite loci showed that the backbone structure of the microsatellite cluster analysis was found to be represented by the microsatellite loci CNA3B, CNA3C and CNA4C that all had a low genotypic diversity and low discriminatory power. Statistical analysis revealed no significant differences between the MCs when compared to the clinical origin, date of isolation, AFLP genotype or geographic locality.


A
MC1
MC4
MC2
MC11
MC14
MC5
MC3
MC8
MC6
MC7
MC13
B
C

Previous page, Figure 1. Population structure of *C. neoformans* var. *grubii* isolates based on serotype A specific microsatellite typing. Panel A shows the microsatellite clusters (MCs) that are numbered according to Illnait-Zaragozí and colleagues (2010), MC13 and MC14 are novel MCs not observed in previous microsatellite typing studies. Panel B is identical to A, but now colors represent the AFLP genotype of the isolates, green = AFLP1, dark blue = AFLP1B, red = AFLP3. Panel C is identical to B, but now colors represents source of isolation with red s = CSF, green = blood, dark blue = other source than blood or CSF, yellow = unknown source.

Microsatellite genotyping of serotype D isolates

A set of 53 *C. neoformans* isolates that included 36 serotype D (AFLP2), 14 serotype AD (AFLP3) and three serotype BD (AFLP8) isolates (Table 3), was studied by serotype D specific microsatellite analysis. When all seven microsatellite CND markers were combined, 32 different microsatellite profiles and four MCs could be distinguished among 53 isolates (Figure 2). The discriminatory power for the complete set of seven microsatellite markers was found to be 0.966. Among the seven studied microsatellite loci, CND2A and CND2B had the highest genotypic diversity with nine alleles (*D*=0.813 and 0.730, respectively), while locus CND3C had the lowest genotypic diversity and the lowest resolution with three alleles (*D*=0.466). There were no cross reactions observed when the serotype D microsatellite typing was performed on a set of non-serotype D *C. neoformans* and *C. gattii* isolates (data not shown). Statistical analysis revealed no significant differences between the MCs when compared to the clinical origin, date of isolation, AFLP genotype or geographic locality.

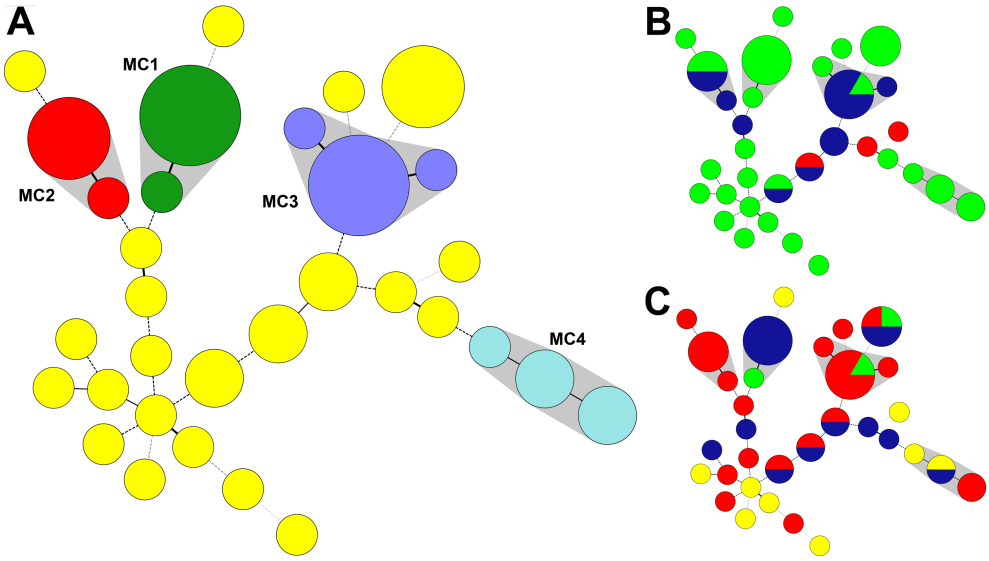


Figure 2. Population structure of *C. neoformans* var. *neoformans* isolates based on serotype D specific microsatellite typing. Panel A shows the four microsatellite clusters (MCs). Panel B is identical to A, but now colors represent the AFLP genotype of the isolates, green = AFLP2, dark blue = AFLP3, red = AFLP8. Panel C is identical to B, but now colors represents source of isolation, red = CSF, green = blood, dark blue = other source than blood or CSF, yellow = unknown source.

Mixed* C. neoformans *infections determined by AFLP and microsatellite typing
Multiple isolates were available from 38 patients. The majority of these isolates were sampled on the same day but from different body sources. While microsatellite profiles of most of these isolates were found to be identical, a few showed minor differences of one to three repeat units' difference between one and two of the nine studied microsatellite loci.

Some patients were found to be infected with multiple *C. neoformans* strains. One immunosuppressed patient yielded nine isolates during a period of two weeks, and two showed major differences in three out of the nine microsatellite loci studied. A three loci difference was also observed between isolates sampled on the same day from CSF and blood of one patient. The two isolates obtained from a patient that experienced a cryptococcosis relapse were found to be different for six loci and were a mix of minor (1 or 2 changes) and major changes (>10) in repeat lengths. This pattern of minor and major difference in repeat lengths was also observed between two isolates sampled on the same day from a patient that was also found to be infected with *C. neoformans* serotype D, which was isolated 21 days prior and 24 days after the isolation of both serotype A isolates. These two serotype D isolates were found to belong to two different genotypes of *C. neoformans* var. *neoformans* based on microsatellite typing since they differed for six of the seven microsatellite loci. Two interspecies hybrid isolates of *C. neoformans* var. *neoformans* and *C. gattii* (AFLP8) were cultured with an 88 days interval from an immunocompromised patient and were found to differ for five of the seven serotype D microsatellite loci.

In vitro *susceptibility profiling*
Minimal inhibitory concentration (MIC) ranges, MIC_{50}s, MIC_{90}s and geometric mean MICs of seven antifungal compounds are presented in Table 4. Susceptibility data for each of the seven antifungal compounds is presented for all 300 *Cryptococcus* spp. isolates as well as for the three major genotypes AFLP1 (n=245), AFLP2 (n=36) and AFLP3 (n=14). For all these four groups, the MIC_{50} and the geometric mean MIC values differed less than one $\log_2$ dilution step, except for fluconazole and itraconazole.

The overall MIC ranges for each of the seven antifungal compounds was 0.063 – 1 µg/ml for amphotericin B, 0.125 – >64 µg/ml for 5-flucytosine, 0.25 – 64 µg/ml for fluconazole, 0.016 – 0.5 µg/ml for itraconazole and posaconazole, 0.016 – 1 µg/ml for voriconazole and <0.016 – 0.5 µg/ml for isavuconazole. Fluconazole and 5-flucytosine had the highest geometric mean MICs of 2.865 and 3.505 µg/ml, respectively, while amphotericin B (0.242 µg/ml), itraconazole (0.082 µg/ml), voriconazole (0.071 µg/ml), posaconazole (0.058 µg/ml) and isavuconazole (0.033 µg/ml) had much lower geometric mean MIC values. The 5-flucytosine resistant *Cryptococcus* isolate (MIC >64 µg/ml) was cultured from blood obtained from a HIV-positive patient. However, a second CSF isolate from this patient from the same day was found to be susceptible (MIC 8 µg/ml) while microsatellite typing revealed that both isolates were nearly identical. Less susceptible (≥16 µg/ml) for 5-flucytosine and fluconazole were nine and ten *C. neoformans* isolates, respectively.

Table 4. Antifungal susceptibility per *C. neoformans* AFLP genotype-cluster. Range, MIC_{50}, geometric mean MIC and MIC_{90} are listed for the tested antifungal compounds for each group.

Origin (number of isolates)	Drug	MIC (µg/ml)			
		Range	MIC_{50}	Geometric mean	MIC_{90}
C. neoformans* var. *grubii	Amphotericin B	0.125 - 1	0.25	0.259	0.5
Serotype A; AFLP1 (*n*=245)	5-Flucytosine	0.125 - >64	4	3.300	8
	Fluconazole	0.25 - 64	4	3.101	8
	Itraconazole	0.016 - 0.5	0.125	0.093	0.25
	Voriconazole	0.016 - 1	0.063	0.076	0.125
	Posaconazole	0.016 - 0.5	0.063	0.063	0.125
	Isavuconazole	<0.016 - 0.5	0.031	0.035	0.125
C. neoformans* var. *neoformans	Amphotericin B	0.063 - 1	0.125	0.180	0.25
Serotype D; AFLP2 (*n*=36)	5-Flucytosine	0.5 - 64	4	5.238	16
	Fluconazole	0.25 - 16	1	1.387	8
	Itraconazole	0.016 - 0.5	0.031	0.041	0.25
	Voriconazole	0.016 - 0.5	0.031	0.040	0.125
	Posaconazole	0.016 - 0.25	0.031	0.035	0.125
	Isavuconazole	<0.016 - 0.25	0.031	0.023	0.125
C. neoformans* var. *neoformans	Amphotericin B	0.063 - 1	0.125	0.160	0.25
Serotype AD; AFLP3 (*n*=14)	5-Flucytosine	2 - 8	4	3.807	8
	Fluconazole	1 - 32	4	4.203	8
	Itraconazole	0.031 - 0.5	0.063	0.066	0.125
	Voriconazole	0.031 - 0.25	0.063	0.080	0.125
	Posaconazole	0.016 - 0.125	0.063	0.047	0.063
	Isavuconazole	0.016 - 0.25	0.031	0.038	0.063
C. neoformans* and *C. gattii	Amphotericin B	0.063 - 1	0.25	0.242	0.5
All genotypes (*n*=300)	5-Flucytosine	0.125 - >64	4	3.505	8
	Fluconazole	0.25 - 64	4	2.865	8
	Itraconazole	0.016 - 0.5	0.125	0.082	0.25
	Voriconazole	0.016 - 1	0.063	0.071	0.125
	Posaconazole	0.016 - 0.5	0.063	0.058	0.125
	Isavuconazole	<0.016 - 0.5	0.031	0.033	0.125

Patients from whom multiple *C. neoformans* isolates were cultured, either at the same time from different body sites or at different time points, showed in general the same pattern of MIC values for the seven tested antifungal compounds. However, two HIV-positive patients were infected with serotype A isolates that showed large differences in fluconazole susceptibility (MICs of 4 and 64 µg/ml) while the isolates were genetically nearly all identical with a minor difference for one microsatellite locus.

Statistical analysis showed that genotype AFLP1 (serotype A) isolates were significantly less susceptible to amphotericin B (0.259 µg/ml) than isolates with genotype AFLP2 (serotype D) and AFLP3 (serotype AD) (0.180 and 0.160 µg/ml, respectively) (P<0.0001). AFLP2 isolates were found to be significantly (P=0.0030) less susceptible to 5-flucytosine (5.238 µg/ml) than AFLP1 and AFLP3 isolates (3.300 and 3.807 µg/ml, respectively). AFLP2 isolates were significantly more susceptible to fluconazole, itraconazole, voriconazole, posaconazole (P<0.0001 for each) and isavuconazole (P=0.0022) than AFLP1 and AFLP3 isolates, as shown by their geometric mean MIC values (Table 4). However, based on the geometric mean MIC values, only those of genotype AFLP2 isolates were found to differ more than a $\log_2$ dilution step for fluconazole when compared to AFLP1 and AFLP3 isolates, whereas genotype AFLP2 isolates also differed a $\log_2$ dilution step for itraconazole compared to genotype AFLP1 isolates. No significant differences in antifungal susceptibility patterns were observed between the four patient categories.

Discussion

A cohort of Dutch HIV-positive cryptococcosis patients has been investigated previously from a clinical epidemiological point of view, but no data were presented on the *Cryptococcus neoformans* isolates (Van Elden *et al*., 2000). To obtain a detailed insight into the epidemiology of this opportunistic pathogenic yeast in the Netherlands, a set of 300 *C. neoformans* isolates from 237 patients obtained during the period 1977 to 2007 was investigated.

The majority of cryptococcosis patients were found to be HIV-positive (n=115; 48.5%). When patients with a known immune status were considered, the percentage of HIV-positive patients was 62.8%, which is similar to data from other European studies. A French study showed that 77% (n=177) of the patients with cryptococcosis were HIV-infected (Dromer *et al.*, 2007), in a cohort of 77 Austrian, German and Swiss patients this was 68% (n=52) (Tintelnot *et al.*, 2004), a Spanish study observed 87% (n=48) (Guinea *et al.*, 2010) and a 30-month European survey reported 77% (n=435) of HIV-positive patients (Viviani *et al.*, 2006). In the current study, 18 (7.6%) patients were found to have no underlying disease or any other predisposing factor, such as corticosteroid treatment. When corrected to patients with a known immune status the percentage of immunocompetent patients was found to be 9.9%. A French study observed nine (4%) immunocompetent patients within the studied cohort (Dromer *et al.*, 2007). Remarkably, several recent reports from Far-East Asia noted that large numbers of cryptococcosis patients are immunocompetent, with values ranging from

19% to 92% of the investigated cohort of patients from China, South Korea, Taiwan and Vietnam (Chau *et al.*, 2010; Choi *et al.*, 2010; Day *et al.*, 2011; Liaw *et al.*, 2010; Zhu *et al.*, 2010). Interestingly, these immunocompetent patients were found to be infected with *C. neoformans* var. *grubii*, which was also the case for the majority (*n*=20; 83.3%) of Dutch immunocompetent cryptococcosis patients. Recent studies from South Korea and Vietnam have observed a correlation between the immune status of the patient and the genotype of the cultured *C. neoformans* var. *grubii* isolate (Choi *et al.*, 2010; Day *et al.*, 2011).

In contrast to *C. neoformans* var. *grubii*, the epidemiology of *C. neoformans* var. *neoformans* has been poorly studied. From few available studies it is known that serotype D isolates are more prevalent in Europe (Kwon-Chung & Bennett, 1984; Viviani *et al.*, 2006). In the current study, 12.7% of the clinical isolates were serotype D (Table 3). A higher percentage of isolates (20.5%) was found among 410 French *C. neoformans* isolates (Dromer *et al.*, 1996). However, in Italy, 71% of the *C. neoformans* isolates were found to be serotype D (Tortorano *et al.*, 1997). A European survey observed that a large proportion of the 311 *C. neoformans* isolates were serotype D (30%) and serotype AD (19%) (Viviani *et al.*, 2006). Thus the Mediterranean area has a higher proportion of infections caused by *C. neoformans* var. *neoformans* when compared to the North Western European area (Guinea *et al.*, 2010; Viviani *et al.*, 2006). Furthermore, genotyping studies revealed that 33.9% of the isolates from Madrid, Spain, fell into the hybrid genotype cluster AFLP3 (serotype AD) and 19.6% in genotype AFLP2 (serotype D) (Guinea *et al.*, 2010). Other epidemiological surveys from Brazil, China, South Korea, Taiwan and Vietnam revealed that serotype D isolates are rarely found, if not absent (Barreto de Oliveira *et al.*, 2004; Choi *et al.*, 2010; Liaw *et al.*, 2010).

Until recently, it was believed that *C. neoformans* had a predilection to cause disease in immunocompromised patients while *C. gattii* was found to preferentially cause disease in immunocompetent individuals (Bovers *et al.*, 2008a). However, several studies observed that a substantial number of cryptococcal infections in African HIV-infected patients are caused by *C. gattii*, thus indicating that the presumed preference for a certain patient category does not seem valid (Morgan *et al.*, 2006; Steele *et al.*, 2010). In the Netherlands, two patients were found to be infected by *C. gattii*. One case was caused by a travel-related *C. gattii* AFLP6 infection in an immunocompromised patient who developed cryptococcosis after corticosteroid treatment (Hagen *et al.*, 2010b). The second case was caused by a *C. gattii* AFLP4 isolate that was cultured from a 54-year-old male who became HIV-infected during his stay in Africa. MLST analysis revealed that this latter isolate fell into a clade with African *C. gattii* AFLP4 isolates (F. Hagen and T. Boekhout, unpublished data). These cases suggest that *C. gattii* can be dormantly present in the human host and that it can be activated when the immune status is attenuated due to disease or treatment with corticosteroids. Three *Cryptococcus* isolates from the current study were found to be interspecies hybrids between *C. gattii* AFLP4 and *C. neoformans* AFLP2 and caused disease in an apparently healthy and an immunocompromised patient, respectively (Bovers *et al.*, 2006).

Microsatellite typing has recently been applied to *C. neoformans* var. *grubii* and has shown to be an excellent tool to discriminate isolates as well as to study their

epidemiology (Illnait-Zaragozí *et al.*, 2010, 2011). This molecular typing tool was first applied using a set of 122 clinical and 68 environmental *C. neoformans* var. *grubii* isolates from Cuba and distinguished 104 different genotypes (D_{CUBA}=0.993) and eleven microsatellite clusters (MCs). The genetic diversity of the Dutch clinical *C. neoformans* var. *grubii* population is higher than that observed in Cuba (D_{NL}=0.994) with 196 genotypes among 259 studied isolates, including the two clusters MC13 and MC14 (Figure 1). The higher genetic diversity of the Dutch *C. neoformans* var. *grubii* population when compared to the Cuban populations may be due to one or more of the following. Firstly, the time span of this retrospectively study is 30 years, which is longer than the 20-year time frame of the Cuban study. Secondly, the composition of the Dutch population is characterized by extensive immigration, which includes people from the former overseas colonies of Surinam, Netherlands Antilles and Indonesia as well as immigrants from Northern Africa and Eastern Europe (CBS Statistics Netherlands; http://www.cbs.nl/; population data 1899-2010). It is likely that a proportion of the Dutch cryptococcosis patients are immigrants that acquired a subclinical infection with *C. neoformans* prior to immigration to the Netherlands, and this may contribute to the observed higher genetic diversity in the studied cohort. Thirdly, it has recently been shown that *C. neoformans* can produce *in vivo* diploid offspring from haploid parental isolates, either via cell fusion or endoreplication (Desnos-Ollivier *et al.*, 2010). Thus a further possibility is that dormant *C. neoformans* isolates become activated upon immune suppression and that during the course of infection and/or antifungal therapy *C. neoformans* cells fuse to form hybrids that might be more resistant against the antifungal compounds (Desnos-Ollivier *et al.*, 2010). The recent observation of clinical interspecies hybrids between *C. neoformans* and *C. gattii* from clinical sources suggests that these hybrids can be formed *in vivo* (Bovers *et al.* 2006, 2008c).

Antifungal susceptibility testing has frequently been applied for *C. neoformans*. The development of new antifungal drugs, such as the novel triazoles and the reported increase in antifungal resistance, highlights the importance of monitoring the susceptibility profiles of clinical *C. neoformans* isolates. The number of resistant and less susceptible isolates observed in the current study remained low. One isolate was found to be 5-flucytosine resistant and nine were less susceptible for this antifungal. Nine isolates were less susceptible for fluconazole, while all other antifungal compounds inhibited the growth of *C. neoformans*. A global antifungal susceptibility study revealed that the observed resistance against amphotericin B, 5-flucytosine and fluconazole was less than one percent of the 1,811 tested isolates (Pfaller *et al.*, 2005). Interestingly, these authors found that, during the time span of their study, *C. neoformans* became more susceptible for 5-flucytosine and fluconazole. This phenomenon, however, has not been observed in the current study. It seems that the number of *C. neoformans* isolates resistant against the conventional antifungal compounds remains limited. Several recently published antifungal susceptibility studies, including the current, showed that posaconazole, voriconazole and isavuconazole are promising candidates to substitute the conventional drugs since the latter have toxic side effects and needs to be administered in higher doses (this study; Guinea *et al.*, 2010; Illnait-Zaragozí *et al.*, 2008; Pfaller *et al.*, 2005; Thompson *et al.*, 2009). The present study shows that genotype

AFLP1 and AFLP3 isolates are less susceptible to fluconazole than AFLP2 isolates. This genotypic difference was only recently observed in a Croatian study that found a similar result for fluconazole, although this difference was not significant between AFLP2 and AFLP3 isolates (Mlinarić-Missoni *et al.*, 2011). The current study shows that there is a trend towards lower MIC values for genotype AFLP2 isolates against amphotericin B similar as observed in the Croatian and Spanish study (Guinea *et al.*, 2010; Mlinarić-Missoni *et al.*, 2011). The Croatian and Spanish study did not observe a significant difference between any of the tested antifungal compounds versus the AFLP genotypes, except for Spanish AFLP1 isolates that were less susceptible to amphotericin B than Spanish AFLP3 isolates (Guinea *et al.*, 2010; Mlinarić-Missoni *et al.*, 2011). Similar to our study, the Spanish study did not observe a significant difference between patient categories versus antifungal susceptibility outcome.

In conclusion, *C. neoformans* var. *grubii* is the major cause of cryptococcosis among immunocompromised and immunocompent individuals in the Netherlands. AFLP genotyping and microsatellite typing showed that the Dutch clinical *C. neoformans* var. *grubii* population is genetically highly diverse compared to other recently studied populations. *In vitro* antifungal susceptibility testing showed that resistance and decreased susceptibility is not yet a major issue in the Netherlands and the novel triazoles are promising candidates for treatment strategies against cryptococcosis.

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Chapter 3

Antifungal susceptibility, serotyping, and genotyping of clinical *Cryptococcus neoformans* isolates collected during 18 years in a single institution in Madrid, Spain

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Summary

We studied the serotypes, mating-types, AFLP genotypes, and antifungal susceptibility of 58 *Cryptococcus neoformans* isolates causing 56 episodes of cryptococcosis in 55 patients over an 18-year period in a single institution. The underlying conditions of the patients were classified as HIV infection (*n*=48) or non–HIV-related immunodeficiency (*n*=7). Serotype A (*n*=3; 58.9%) predominated, but serotype AD was involved in 23.2% of episodes. Most of the episodes were caused by mating-type α (*n*=41; 73.2%) or α/**a** isolates (*n*=12; 21.5%). The most common genotype was AFLP1 (*n*=26; 46.4%), followed by AFLP3 (*n*=21; 37.5%), and AFLP2 (*n*=11; 19.6%). In two different patients, we showed the coexistence of different serotypes and/or genotypes in the same episode (AFLP1 and 3). The new triazoles voriconazole, posaconazole and isavuconazole showed high and similar antifungal activity (MICs ≤0.125 μg/ml). Fluconazole also had good antifungal activity, only two isolates from patients with HIV-infection showed an MIC of 16 μg/ml (3.4%), and they remained very susceptible to the new triazoles (MICs ≤0.062 μg/ml). The remaining isolates always showed MICs ≤8 μg/ml.

Introduction

Cryptococcosis is an opportunistic fungal infection caused by *Cryptococcus neoformans* or *Cryptococcus gattii.* The latter is more prevalent in immunocompetent individuals, while infection by *C. neoformans* mainly affects immunocompromised patients (Morgan *et al.*, 2006; Singh *et al.*, 2008). The taxonomy of the *C. neoformans*/*C. gattii* complex is currently under review, but it includes at least two pathogenic members: *C. gattii* and *C. neoformans* (Kwon-Chung *et al.*, 2002; Kwon-Chung & Varma, 2006). *Cryptococcus neoformans* is classified into three varieties: *C. neoformans* var. *grubii* (serotype A; genotype AFLP1), *C. neoformans* var. *neoformans* (serotype D; genotype AFLP2), and hybrids of both varieties (serotype AD; genotype AFLP3) (Bovers *et al.*, 2006, 2008a; Kwon-Chung *et al.*, 2002). *Cryptococcus gattii* strains belong to serotypes B or C (genotype AFLP4, 5, 6, and 7). Interspecies hybrids of *C. gattii* × *C. neoformans* var. *neoformans* (serotype BD; genotype AFLP8) and of *C. gattii* × *C. neoformans* var. *grubii* (serotype AB; genotype AFLP9) have recently been described (Boekhout *et al.*, 2001; Bovers *et al.*, 2006, 2008b). Genotyping of *Cryptococcus* spp. strains allows us to determine whether cryptococcosis is caused by a single genotype or by co-infection with two or more genotypes.

The recent introduction of new antifungal agents means that the antifungal susceptibility profile of clinical *C. neoformans* strains must be updated. Our study analyzes the agents causing cryptococcosis over the last 18 years in a large teaching institution. We assess the phenotypic and genotypic characteristics and antifungal susceptibility profiles of the strains.

Materials and methods

Episodes, patients, and C. neoformans *isolates*

From 1990 to 2007, we detected 70 episodes of cryptococcosis in 68 different patients in our hospital. Episodes were considered different when isolation of *Cryptococcus* spp. was separated by >3 months. We analyzed a subset of 56 episodes (55 patients) for which isolates were available. Cryptococcosis presented as meningitis (n=24), fungemia plus meningitis (n=21), and fungemia alone (n=11). The underlying conditions of the patients were classified as HIV infection (n=48) or non–HIV-related immunodeficiency (n=7). The patients with non-HIV-related immunodeficiency had received a solid organ transplant (n=4, [3 renal transplant recipients and 1 heart transplant recipient]) or had other immunosuppressive conditions (n=3, [1 HCV cirrhosis; 1 rheumatoid arthritis under immunosuppressive therapy with corticosteroids, methotrexate, and infliximab; 1 systemic lupus erythematosus and alveolar proteinosis]).

A total of 95 available *C. neoformans* isolates from 55 patients were studied. Samples from patients were taken when clinically indicated. Only one colony from each plate was stored. The number of isolates per patient was not homogeneously distributed: 35 patients had only 1 isolate, 9 patients had 2 isolates, 6 patients had

3 isolates, 3 patients had 4 isolates and 2 patients had 6 isolates. The *Cryptococcus* spp. isolates were stored at -70°C in tubes containing sterile distilled water. To ensure viability and purity, each isolate was subcultured on sheep blood agar (Soria Melguizo, Madrid, Spain) before analysis. In patients with multiple isolates available from the same episode, only the first isolate of each genotype was selected (58 isolates).

Genotyping, serotyping, and mating-typing

The *Cryptococcus* isolates were analyzed for their mating-, sero-, and genotype and underwent antifungal susceptibility testing. All available isolates from each patient were genotyped in order to detect co-existence of different genotypes in a single episode. The mating-types and serotypes of the isolates were determined using four different polymerase chain reactions (PCRs) that specifically amplify the *STE20***a** and *STE20*α locus of either serotype A and D isolates (Barreto de Oliveira *et al.*, 2004).

Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis was carried out to determine the genotypes of all *C. neoformans* isolates (Boekhout *et al.*, 2001; Bovers *et al.*, 2008b). The strains CBS8710 (AFLP1/VNI; αA), CBS9172 (AFLP1/VNI; **a**A), CBS10511 (AFLP2/VNIV; αD), CBS10513 (AFLP2/VNIV; **a**D), CBS10078 (AFLP4/VGI; αB), CBS10080 (AFLP3/VNIII; αA**a**D), CBS10081 (AFLP5/VGIII; αB), CBS10082 (AFLP6/VGII; αB), and CBS10101 (AFLP7/VGIV; αC) were included as quality control strains for mating-, sero-, and genotyping. A dendrogram was calculated using single linkage clustering in combination with the Pearson correlation using Bionumerics v4.6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Antifungal susceptibility testing procedures

A total of 58 isolates were available for antifungal susceptibility testing. We used the antifungal agents obtained as reagent-grade powders from their respective suppliers. The panel of five antifungal agents included amphotericin B (Sigma Chemical Co., Madrid, Spain), fluconazole, voriconazole (Pfizer Pharmaceutical Group, New York, NY), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), and posaconazole (Schering-Plough Research Institute, Kenilworth, NJ). Antifungal activity was determined using the CLSI M27-A3 broth microdilution procedure (Clinical and Laboratory Standards Institute, 2008). The final concentrations for each antifungal agent were as follows: amphotericin B, posaconazole, isavuconazole, and voriconazole, 0.031 μg/ml to 32 μg/ml; and fluconazole 0.062 μg/ml to 64 μg/ml. No precipitates were observed for concentrations of posaconazole above 8 μg/ml.

Although the CLSI M27-A3 procedure recommends that inocula be prepared after culturing isolates in Sabouraud dextrose agar or potato dextrose agar, we cultured the *C. neoformans* isolates in sheep blood agar for 48-72 hours at 35°C to improve growth. All the inoculated trays were incubated at 35°C and read macroscopically at 72 hours. The MIC endpoint for amphotericin B was defined as the lowest concentration that produced complete inhibition of growth (MIC-0), whereas for the remaining agents it was defined as the lowest concentration at which a prominent decrease in turbidity, corresponding to approximately 50% inhibition of growth, was observed (MIC-2) (Clinical and Laboratory Standards Institute, 2008). The same batch of trays

was used to assess antifungal susceptibility. Quality control was ensured by testing *Candida krusei* ATCC6258 and *Candida parapsilosis* ATCC22019, and all results were within the recommended CLSI limits. The activity of the five antifungal agents was shown as the MIC_{50}, MIC_{90}, and range of MICs.

Data analysis

We analyzed the correlation between the presence of a specific different mating-type, serotype, or genotype of *C. neoformans*, and the predisposing conditions of the patients or their sex (Fisher exact test). The activity of the five antifungal agents was shown as the cumulative percentage of isolates in each MIC and geometric mean (GM). For calculation of GM, MICs below the lowest antifungal concentration were considered 0.015 μg/ml (for the new triazoles). We analyzed the differences in the GM of the MICs of the antifungal agents against the isolates grouped by mating-type, serotype, and genotype, and predisposing condition (HIV infection or other immunosuppressive conditions). The null hypothesis was rejected in every hypothesis contrast for an α below 0.05.

Results

Mating-, sero-, and genotyping

Tables 1 and 2 and Figure 1 show the distribution of serotypes, mating-types, and genotypes of the 58 isolates causing the 56 episodes of cryptococcosis. Serotype A (*n*=3; 58.9%) predominated, although serotype AD was involved in 23.2% of the episodes. The mating-type analysis revealed that most of the episodes were caused by α (*n*=41; 73.2%) or α/**a** isolates (*n*=12; 21.5%). One episode was caused by a *C. neoformans* isolate that was non-typeable for the mating-type and the serotype, but was identified based on AFLP genotyping as AFLP1B, corresponding to *C. neoformans* var. *grubii*.

The most widely distributed AFLP genotype was AFLP1 (*n*=26; 46.4%), followed by AFLP3 (*n*=21; 37.5%) and AFLP2 (*n*=11; 19.6%). In 35 episodes (35 patients), only one available isolate was analyzed and no genetic diversity was identified. However, in the remaining 21 episodes (20 patients) we were able to analyze two or more isolates

Table 1. Serotype, mating-type, and AFLP genotype of 58 *Cryptococcus neoformans* isolates causing 56 episodes of cryptococcosis.

	Serotype[1]					Mating-type[2]					AFLP genotype[3]			
	A	D	AD	A and AD	Non-typeable	α	α/a	a	α/α	α and α/a	Non-typeable	1	2	3	1 and 3
Episodes (*n*=56)	31 (55.3%)	11 (19.6%)	11 (19.6%)	2 (3.6%)	1 (1.8%)	39 (69.6%)	10 (17.9%)	3 (5.4%)	1 (1.8%)	2 (3.6%)	1 (1.8%)	24 (42.8%)	11 (19.6%)	19 (33.9%)	2 (3.6%)

[1] One strain was non-typeable and two episodes were caused by a combination of the A and AD serotypes; [2] Two episodes were caused by a combination of the and α/**a** mating-types; [3] Two episodes were caused by a combination of strains with genotypes AFLP1 and AFLP3.

Figure 1. AFLP genotyping, mating- and serotyping of *Cryptococcus neoformans* strains

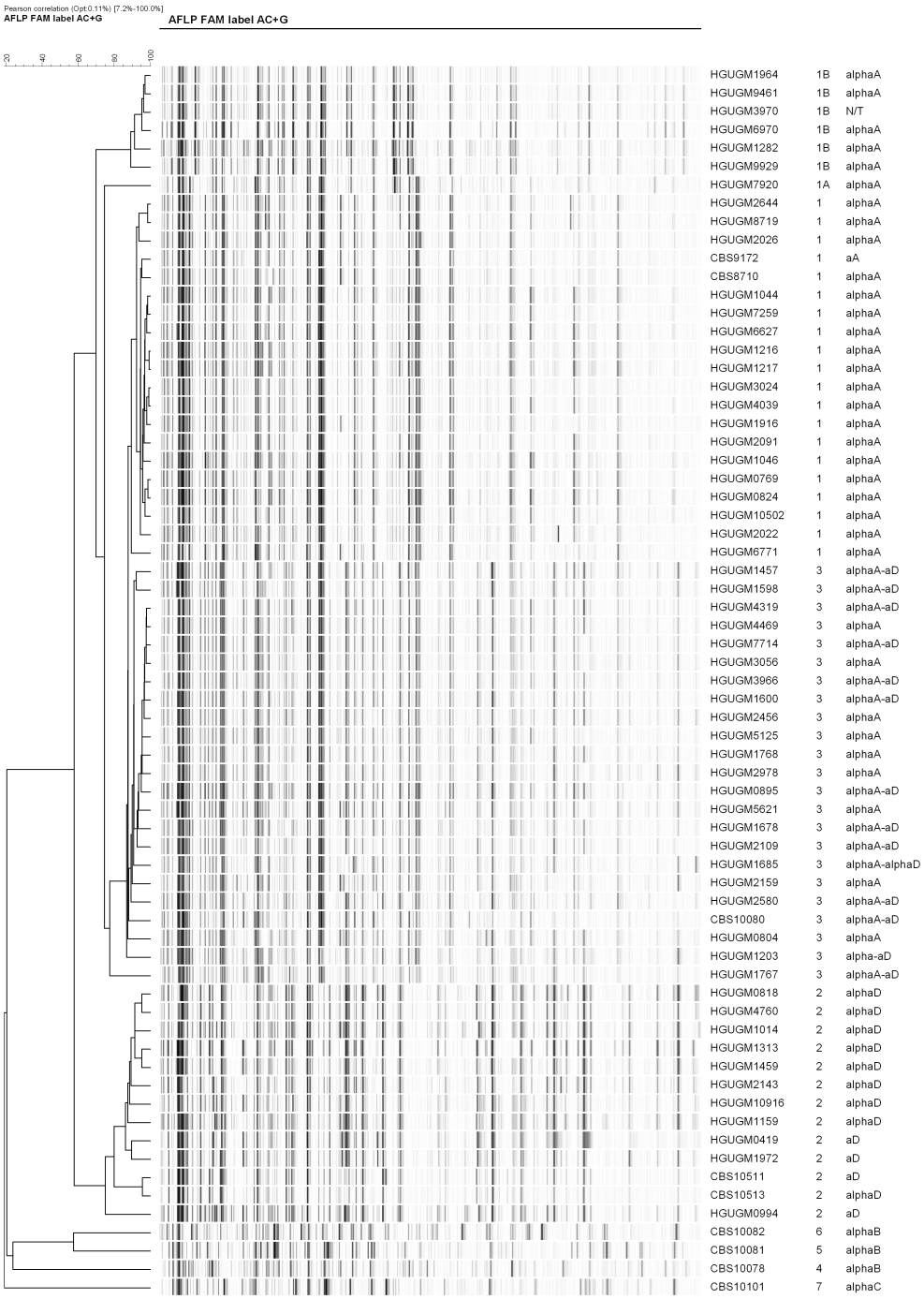

Table 2. Relationship between the different mating-types, serotypes, and genotypes of *Cryptococcus neoformans* and the sex and underlying conditions of the patients

		Underlying condition[1]			Sex[2]		
	n	**HIV (%)**	**Other (%)**	***P*-value**	**Female (%)**	**Male (%)**	***P*-value**
Serotype[3]							
A	33	54.9	83.3	0.384	90	51.1	0.034
D	11	21.6	0	0.584	0	23.4	0.183
AD	13	23.5	16.7	1.000	10	25.5	0.426
Mating-type[3, 4]							
α	41	70.6	83.3	0.665	90	68.1	0.253
α/a	12	21.6	16.7	1.000	10	23.4	0.671
AFLP genotype							
1, 1A or 1B	26	85.7	39.2	0.038	90.9	34	0.001
2	11	21.6	0	0.327	23.4	0	0.102
3	21	39.2	14.3	0.403	9.1	42.6	0.044

[1] Percentage of patients with HIV infection or other immuno-deficiencies infected by each mating, sero- and AFLP genotype; [2] Percentage of females or males infected by each mating, sero- and AFLP genotype; [3] One strain was non-typeable; [4] Mating-types **a** (*n*=3) and **α/α** (*n*=1) were not included in this analysis.

per episode, and this allowed us to demonstrate the co-existence of different serotypes and/or genotypes in the same episode in two different patients. One HIV-infected patient had an episode caused by two different *C. neoformans* genotypes (AFLP1 and 3); this was consistent with the results of the mating-type and serotype analyses (two αA isolates were cultured from cerebrospinal fluid and blood, and one αA**a**D isolates was isolated from a cerebrospinal fluid sample taken three weeks later). Another HIV-infected patient also had an episode caused by two different genotypes (an AFLP1B isolate and an AFLP3 isolate, both cultured from cerebrospinal fluid samples taken with an interval of 15 days).

In our hospital, episodes of cryptococcosis were caused by *C. neoformans* var. *grubii* (*n*=24; 42.9%), *C. neoformans* var. *neoformans* (*n*=11; 19.6%), the hybrid *C. neoformans* var. *grubii* × *C. neoformans* var. *neoformans* (*n*=19; 33.9%), and the co-existence of both *C. neoformans* var. *grubii* and the hybrid *C. neoformans* var. *grubii* × *C. neoformans* var. *neoformans* (*n*=2; 3.6%).

Twenty-two isolates belonged to genotype AFLP3, 13 of these were serotype AD by PCR analysis, while of the remaining nine isolates only the serotype A background could be determined. Serotype AD hybrids may become aneuploid, with the result that

Table 3. Cumulative percentages of *Cryptococcus neoformans* isolates within each MIC (µg/ml) of the five antifungal agents studied

Antifungal agents	≤0.031	0.062	0.125	0.25	0.5	1	2	4	8	16
Isavuconazole	89.7	96.6	100							
Posaconazole	96.6	100								
Voriconazole	75.4	94.7	100							
Fluconazole					3.45	22.8	52.6	78.9	96.5	100
Amphotericin B					34.5	96.5	100			

the full serotype AD background is no longer present in the genomic DNA of these isolates.

Relationship between isolates and clinical data

We could not find a specific mating-type or serotype that was more prevalent in HIV-infected patients than in patients with non-HIV-related immunodeficiency. However, we found that the genotypes AFLP1, 1A, and 1B were more prevalent in HIV-infected patients (P=0.034) (Table 2). Serotype A and genotypes AFLP1, 1A, and 1B isolates were more prevalent in females, whereas genotype AFLP3 isolates were more prevalent in males (P<0.05) (Table 2).

Antifungal susceptibility testing results

Table 2 summarizes the *in vitro* antifungal susceptibility results of the 58 isolates involved in the 56 episodes of cryptococcosis. The new triazoles voriconazole, posaconazole, and isavuconazole showed high and similar antifungal activity, all isolates had an MIC ≤0.125 µg/ml. Fluconazole also had good antifungal activity, only two isolates from HIV-infected patients had a MIC of 16 µg/ml (3.4%) and both remained highly susceptible to the three new triazoles (MICs ≤0.062 µg/ml). The remaining isolates always showed MICs ≤8 µg/ml. Only two isolates showed an MIC >1 µg/ml for amphotericin B.

We did not observed differences in antifungal susceptibility between isolates isolated from HIV-infected patients and non-HIV-infected patients.

We compared the geometric mean of the MICs of each antifungal agent against isolates grouped by serotype, mating-type and AFLP genotype. Isolates belonging to serotype A showed significantly higher MICs (0.937 µg/ml) for amphotericin B than those from serotype D (0.818 µg/ml) and serotype AD (0.692 µg/ml) (P<0.01). Isolates belonging to genotype AFLP1, 1A, and 1B were less susceptible for amphotericin B (0.981 µg/ml) than genotype AFLP3 (0.725 µg/ml) (P<0.05). No differences in susceptibility to the new triazoles were observed for the different serotypes, mating-types, and genotypes of *C. neoformans* studied.

Discussion

Our study evaluates the epidemiology of *C. neoformans* in a single institution over a period of 18 years. Cryptococcosis patients admitted to our hospital were infected mainly by *C. neoformans* var. *grubii* (genotype AFLP1). However, a high proportion of episodes were caused by serotype AD (23.2%) isolates. Many clinical *C. neoformans* isolates from Italy, Spain, Portugal, and Greece have been shown to be serotype AD, thus suggesting a high incidence of serotype AD hybrids in southern Europe (Viviani *et al.*, 2006).

AFLP genotyping was applied to all *Cryptococcus* spp. isolates to identify their variety within the *C. neoformans* complex, and to confirm the serotyping analysis based on four different PCRs. Serotyping based on PCR alone may lead to incorrect identifications: diploid isolates (e.g. serotype AD hybrids) can become aneuploid and lose parts of their genomic content, including regions containing the target for the serotype PCRs. This was observed in some of the *C. neoformans* isolates studied and may explain the discrepancy between PCR-based serotyping (58.9% episodes caused by serotype A isolates and 23.2% of the episodes by serotype AD isolates) and AFLP genotyping (46.4% of the episodes caused by AFLP1 isolates and 37.5% of the episodes by genotype AFLP3 isolates).

The presence of two or more different genotypes of *C. neoformans* causing a single episode of cryptococcosis has been poorly evaluated. The presence of several genotypes can only be detected when more than one sample is analyzed from the same patient. In this study, 21 episodes (20 patients) of cryptococcosis yielded at least two isolates from different samples. Although we only stored one colony from each sample, we were able to show that two of the episodes were caused by two different AFLP genotypes, suggesting that cryptococcosis can be a co-infection of multiple *C. neoformans* genotypes. There are few reports on multiple-genotype *C. neoformans* infections. Haynes and co-workers (1995) described the involvement of different *C. neoformans* genotypes in recurrent cryptococcosis, which was explained by the fact that either the patient was re-infected with a distinguishable *C. neoformans* strain during and/or after antifungal treatment, or that one of the strains causing the primary infection was more persistent than the other. Another explanation is that the karyotype became unstable during infection. This phenomenon was reported by Fries and colleagues (1996), who observed that chromosomal arrangements and/or deletions took place in a murine model. However, it seems unlikely that the karyotype became unstable in the mixed infections described, since this implies that the isolate with the hybrid genotype AFLP3 (serotype AD) has lost approximately half of its genetic content during the infection to become an isolate with genotype AFLP1 (serotype A). Therefore, a mixed infection with two different genotypes of *C. neoformans* seems to be the most plausible explanation.

Although several studies have evaluated the antifungal susceptibility of *C. neoformans* and *C. gattii* (Chen *et al.*, 2000; Dannaoui *et al.*, 2006; Khan *et al.*, 2007; Morgan *et al.*, 2006; Tay *et al.*, 2006; Thompson *et al.*, 2009), the introduction of new

drugs means that data on antifungal susceptibility should be updated. We found a low rate of resistance to fluconazole in our set of 58 *C. neoformans* isolates. There is some evidence that the MICs for fluconazole (≥16 μg/ml) and amphotericin B (≥2 μg/ml) against *C. neoformans* can be a predictor of poor outcome in patients with cryptococcosis (Aller *et al.*, 2000; Lozano-Chiu *et al.*, 1998; Witt *et al.*, 1996). Therefore, all isolates in the present study were classified as susceptible to amphotericin B, and 3.6% were fluconazole-resistant (≥16 μg/ml). The higher MICs of amphotericin B for some serotypes or genotypes may not have any clinical consequences. No history of treatment with fluconazole could be demonstrated in the two patients with isolates showing an MIC of 16 μg/ml for fluconazole. Although some authors found a low level of fluconazole resistance in *C. neoformans* when analyzing a large number of clinical isolates (Brandt *et al.*, 2001; Davey *et al.*, 1998; Pfaller *et al.*, 2005), others reported an increasing proportion of resistant isolates (Perkins *et al.*, 2005; Sar *et al.*, 2004). Interestingly, in 2005, Perkins and co-workers reported that 46.6% (≥16 μg/ml) of the isolates in a collection comprising 317 clinical isolates of *C. neoformans* var. *neoformans* from a Spanish mycology reference laboratory were fluconazole-resistant. This proportion of fluconazole-resistant *C. neoformans* isolates is considerably higher than that observed in the present study (3.6%) and by other Spanish investigators (Torres-Rodríguez *et al.*, 2008). The discrepancies may be explained by variations in the antifungal susceptibility testing procedure (EUCAST vs. CLSI) and a bias in the selection of isolates submitted to the reference laboratory.

The new triazoles studied (voriconazole, posaconazole and isavuconazole) showed potent antifungal activity against all the evaluated isolates, with MICs ≤0.125 μg/ml. These findings are consistent with previous reports (Illnait-Zaragozí *et al.*, 2008; Pfaller *et al.*, 2004, 2009; Sabatelli *et al.*, 2008; Torres-Rodríguez *et al.*, 2008).

In conclusion, we found that most cases of cryptococcosis in our hospital were caused by isolates of *C. neoformans*, with a predominance of *C. neoformans* var. *grubii*. The *C. neoformans* isolates showed a low level of resistance to fluconazole, and were highly susceptible to the new triazoles, including isavuconazole.

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Chapter 4

In vitro antifungal susceptibilities and molecular typing of sequentially isolated clinical *Cryptococcus neoformans* strains from Croatia

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Summary

A collection of 48 clinical *Cryptococcus neoformans* strains from Croatia were retrospectively investigated using *in vitro* antifungal susceptibility testing and molecular biological techniques to determine mating- and serotype by PCR as well as by Amplified Fragment Length Polymorphism (AFLP) genotyping. These strains were obtained from fifteen patients, ten were HIV-negative (66.7%) and five were HIV-positive patients (33.3%). From five patients only one strain was available, while from the other ten patients two to eleven strains were sequentially isolated. Antifungal susceptibility was tested by CLSI M27-A3 broth microdilution method. Serotype A (genotype AFLP1) and serotype D (genotype AFLP2) were both found in six patients (40% each), and serotype AD (genotype AFLP3) in three (20.0%) patients. Mating-type α (*n*=12; 80.0%) predominated and α/**a** hybrids were identified in 20% of patients diagnosed with cryptococcosis. Two AFLP genotypes of *C. neoformans* were isolated during a single episode from one patient. The *in vitro* antifungal MIC_{90} and susceptibility ranges for *C. neoformans* strains were 0.5 (0.031 - 0.5) μg/ml for amphotericin B, 4 (1 - 4) μg/ml for 5-flucytosine and fluconazole, 0.25 (0.031 - 0.5) μg/ml for itraconazole and 0.062 (0.031 - 0.25) μg/ml for voriconazole.

Introduction

Cryptococcosis is a life-threatening systemic fungal infection caused by the opportunistic pathogenic yeast *Cryptococcus neoformans* or its primary pathogenic sibling *C. gattii*. Worldwide, *C. neoformans* is the major cause of cryptococcosis in individuals with an underlying immunological disorder, such as patients that are HIV-infected, those who underwent solid organ transplantation (Bovers *et al*., 2008; Warkentien & Crum-Cianflone, 2010) or have haematological malignancies (Vigouroux *et al.,* 2000; Bassetti *et al*., 2009). It has recently been estimated that the global burden of HIV-associated cryptococcosis approximates one million cases annually (Park *et al*., 2009). In Croatia, from 1985, when HIV was diagnosed for the first time, up until December 2009, nearly 800 HIV-infected persons were registered (http:/www.hzjz.hr/; data accessed December 24th, 2010). Croatia has a centralized system of healthcare for HIV/AIDS patients who are all treated at the University Hospital of Infectious Diseases (UHID) in Zagreb. Since 1998, HIV-positive patients are at this hospital treated with Highly Active Antiretroviral Therapy. Extrapulmonary cryptococcosis is an important opportunistic infection, it was found in 29 (8.7%) of the 332 HIV-positive patients treated at the UHID in the period 1986-2010.

The most frequent and severe clinical presentation of cryptococcosis is a disseminated meningoencephalitis, which is almost uniformly fatal if not diagnosed and treated appropriately. In addition to the traditional antifungal compounds amphotericin B, lipid amphotericin B formulations, 5-flucytosine, fluconazole, itraconazole and their combinations, treatment options for cryptococcosis have been increased by the expanded-spectrum azoles voriconazole and posaconazole (Perfect *et al*., 2010).

The application of molecular biological methods, such as PCR fingerprinting, *PLB1* and *URA5* Restriction Fragment Length Polymorphism (RFLP) fingerprinting, Amplified Fragment Length Polymorphism (AFLP) fingerprinting and Multi-Locus Sequence Typing (MLST), has led to a better insight in the taxonomy and identification of the causative agents of cryptococcosis (Bovers *et al*., 2008; Meyer *et al*., 2009). Within *C. neoformans*, three varieties can be distinguished, var. *grubii* (serotype A; AFLP genotype 1), var. *neoformans* (serotype D; AFLP genotype 2) and a hybrid between these two varieties (serotype AD; AFLP genotype 3). A third variety, *C. neoformans* var. *gattii*, was raised to the species level and was named *C. gattii* (Kwon-Chung *et al*., 2002). *Cryptococcus gattii* (serotypes B and C; AFLP genotypes 4-7 and 10) causes predominantly infection in apparently immunocompetent individuals (Bovers *et al.*, 2008; Hagen *et al*., 2010).

Epidemiological surveys provide a better understanding of the population dynamics of *C. neoformans* and *C. gattii*, as visualised by the ongoing *C. gattii* outbreak (Kidd *et al*., 2004). The epidemiology of *C. neoformans* has been studied intensively in Western and Mediterranean Europe (Desnos-Ollivier *et al.*, 2010; Guinea *et al.*, 2010; Van Elden *et al*., 2000; Viviani *et al*., 2006). However, there is a lack of epidemiological studies on the occurrence of cryptococcosis in Eastern Europe. Therefore, we initiated a retrospective study to investigate the epidemiology of cryptococcosis among Croatian citizens as well as the molecular characterisation and *in vitro* antifungal susceptibility profiles of the obtained *Cryptococcus* strains.

Material and methods

Strains and patients

A total of 48 *C. neoformans* strains, obtained from CSF (*n*=46), blood (*n*=1), and sputum samples (*n*=1) of 15 patients during the period February 2005 to February 2010, were studied retrospectively (Table 1). All cryptococcal infections were diagnosed at the Reference Centre for Diagnostics of Systemic Mycoses (RCDSM) at the Croatian National Institute of Public Health in Zagreb, Croatia. There were five HIV-infected patients, while the number of non-HIV-related immunodeficient individuals was ten. The latter were patients with haematological malignancies (*n*=9) and one patient with idiopathic $CD4^+$ lymphocytopenia. The median age of the patients was 54 years (range 24 - 71). The five HIV-infected patients had a median $CD4^+$ T cell count of 27 per mm^3 (range 15 - 51). Fourteen patients had meningitis and one had cryptococcemia without meningitis. The majority of the patients were treated at UHID (*n*=11), two at the Dubrava Clinical Hospital in Zagreb, and two at the Varaždin General Hospital. The patients from UHID were treated initially with a combination of amphotericin B and fluconazole (*n*=5) or sequential amphotericin B and fluconazole (*n*=4) or fluconazole only (*n*=2). The four HIV-negative patients, hospitalized in two other hospitals, did not received antifungal treatment because cryptococcosis was diagnosed post-mortem.

Samples were taken in accordance with clinical indication. All available strains from each patient were analysed. From five patients one *C. neoformans* strain was present, while from ten patients between two and eleven strains were recovered (Table 1). From nine patients, 46 *Cryptococcus* strains were obtained during a single episode of cryptococcosis. The longest time span between the isolation of the first and last *C. neoformans* strain was 58 days, with an average of 14 days. From one patient, two strains were obtained during two episodes of cryptococcosis which were interrupted with a 130-day period (Table 1).

Strains kept at the Culture Collection of the Reference Centre for Diagnostics of Systemic Mycoses (RCDSM) were stored at -80°C using the Microbank system (Pro-Lab Diagnostics, Richmond Hill, ON). All strains were deposited in the public culture collection of the CBS-KNAW Fungal Biodiversity Centre under accession numbers CBS11956-CBS11997 and CBS11999-CBS12004.

Media and identification

The initial diagnosis of cryptococcosis was made by direct examination of CSF samples with India ink preparation and by detection of capsular antigen in CSF and/or serum samples with the latex agglutination test (Pastorex™ Crypto Plus, Bio-Rad, Marnes-la-Coquette, France).

Cryptococcal isolation was carried out using Sabouraud's glucose agar and Brain-Heart Infusion agar (BD Diagnostics, Franklin Lakes, NJ). The ID32C method (BioMerieux, Marcy l'Etoile, France) and morphology on cornmeal agar (BD Diagnostics, Franklin Lakes, NJ) were used for the identification of *C. neoformans* strains.

Table 1. Clinical data, source and the days since first positive *Cryptococcus* culture, serotype, AFLP genotype and MIC values (μg/ml) of five tested antifungal compounds

CRO-strain	Patient	Sex	HIV-status	Source	Hosp. Day	Serotype	AFLP	AMB*	5FC*	FLU*	ITR*	VOR*
1	1	F	HIV+	CSF	0	αA	1	0.062	1	2	0.25	0.062
2				CSF	1	αA	1	0.062	1	2	0.25	0.062
3				CSF	2	αA	1	0.062	1	2	0.25	0.062
4				CSF	3	αA	1	0.062	1	2	0.25	0.062
5				CSF	4	αA	1	0.062	1	4	0.25	0.062
6				CSF	11	αA	1	0.062	2	4	0.5	0.062
7	2	M	HIV-	CSF	0	αA	1	0.5	1	4	0.5	0.031
8	3	M	HIV-	CSF	0	αD**a**A	3	0.062	2	4	0.125	0.062
9				CSF	130	αD**a**A	3	0.5	4	4	0.125	0.125
10	4	M	HIV-	CSF	0	αD	2	0.125	4	2	0.125	0.031
11				CSF	1	αD	2	0.125	4	2	0.125	0.031
12				CSF	2	αD	2	0.125	4	2	0.125	0.031
13				CSF	3	αD	2	0.125	4	2	0.125	0.031
14	5	M	HIV-	CSF	0	αA**a**D	3	0.062	4	1	0.125	0.062
15	6	M	HIV-	CSF	0	αD	2	0.125	4	1	0.125	0.062
16				CSF	1	αD	2	0.125	4	1	0.125	0.062
17				CSF	2	αD	2	0.125	4	1	0.125	0.062
18				CSF	6	αD	2	0.125	4	1	0.125	0.062
19	7	M	HIV+	CSF	0	αD	2	0.062	1	1	0.031	0.062
20				CSF	1	αD	2	0.062	1	1	0.031	0.062
21				CSF	2	αD	2	0.062	1	1	0.031	0.062
22				CSF	14	αD	2	0.062	2	2	0.125	0.062
23				CSF	21	αD	2	0.125	2	2	0.125	0.062
24				CSF	28	αD	2	0.125	4	2	0.125	0.062
25				CSF	34	αD	2	0.25	4	2	0.125	0.062
26				CSF	39	αD	2	0.25	4	2	0.125	0.062
27				CSF	45	αD	2	0.25	4	2	0.125	0.062
28				CSF	53	αD	2	0.5	4	2	0.125	0.062
29				CSF	57	αD	2	0.5	4	2	0.125	0.062

Table 1. *continued*

30				CSF	0	αA	1B	0.062	2	1	0.125	0.062
31	8	M	HIV+	CSF	1	αA	1B	0.062	2	1	0.125	0.062
32				CSF	11	αA	1	0.125	2	1	0.125	0.062
33	9	F	HIV-	Blood	0	αA	1B	0.031	4	2	0.125	0.031
34	10	M	HIV+	CSF	0	αD	2	0.062	4	1	0.062	0.062
35				Sputum	13	αD	2	0.125	4	1	0.062	0.062
36				CSF	0	αA	1	0.062	4	1	0.062	0.062
37				CSF	1	αA	1	0.062	4	1	0.062	0.062
38	11	F	HIV-	CSF	2	αA	1	0.062	4	1	0.062	0.062
39				CSF	4	αA	1	0.062	4	1	0.062	0.062
40				CSF	5	αA	1	0.062	4	1	0.062	0.062
41	12	M	HIV-	CSF	0	αA	1	0.5	4	4	0.5	0.031
42				CSF	0	αD	2	0.062	4	1	0.062	0.062
43	13	F	HIV-	CSF	1	αD	2	0.062	4	1	0.062	0.062
44				CSF	13	αD	2	0.125	4	1	0.062	0.062
45	14	M	HIV-	CSF	0	αD	2	0.5	4	2	0.062	0.031
46				CSF	0	αA**a**D	3	0.062	4	2	0.062	0.125
47	15	M	HIV+	CSF	12	αA**a**D	3	0.062	4	4	0.125	0.125
48				CSF	18	αA**a**D	3	0.062	4	4	0.125	0.25

* AMB = Amphotericin B; 5FC = 5-Flucytosine; FLU = Fluconazole; ITR = Itraconazole; VOR = Voriconazole

Antifungal susceptibility testing procedures

In vitro antifungal susceptibility profiles of *Cryptococcus* strains were determined by broth microdilution method according to the CLSI M27-A3 guidelines (Clinical and Laboratory Standards Institute, 2008). The antifungal agents were directly obtained from the manufacturers and included amphotericin B and 5-flucytosine (Sigma Aldrich, St. Louis, MO), fluconazole and voriconazole (Pfizer Pharmaceuticals, New York, NY) and itraconazole (Jansen Pharmaceutica, Beerse, Belgium). Briefly, powdered forms of antifungal compounds were dissolved in appropriate solvents to prepare stock solutions following the recommendation of the CLSI M27-A3 protocol. Final antifungal concentrations ranged from 0.062 - 64 µg/ml for fluconazole, 0.031 - 16 µg/ml for amphotericin B and 5-flucytosine, and 0.016 - 8 µg/ml for itraconazole and voriconazole. The test inoculum was adjusted to 1×10^3 CFU/ml.

Microdilution plates were incubated at 35°C for 72 hours and the minimal inhibiting concentrations (MICs) end points were subsequently determined visually as complete growth inhibition for amphotericin B and as prominent decrease in turbidity (≥50%) for the other antifungal agents, compared to that

for the growth control. *Candida parapsilosis* ATCC22019 (= CBS604) and *Candida krusei* ATCC6258 (= CBS573) were included as quality control strains.

According to recent studies, *C. neoformans* strains can be considered resistant when the MIC values for amphotericin B are ≥2 μg/ml, 5-flucytosine ≥32 μg/ml, fluconazole ≥16 μg/ml, and itraconazole and voriconazole ≥1 μg/ml (Fusco Almeida *et al.*, 2007; Guinea *et al.*, 2010; Hagen *et al.*, 2010; Perfect *et al.*, 2010; Pfaller *et al.*, 2005; Souza *et al.*, 2005).

Mating-, sero and genotyping

Four different PCRs, using primers specific for each of the four mating- and serotype combinations (**a**A, αA, **a**D and αD), were performed to determine the mating- and serotype of the *C. neoformans* strains by partly amplification of the *STE20* locus (Barreto de Oliveira *et al.*, 2004). Amplification reactions were carried out in a mixture containing 12.9 μl ddH_2O, 2.0 μl 10× PCR buffer (15 mM $MgCl_2$; pH8.3), 2.0 μl dNTPs (1 mM; Bioline, Brussels, Belgium), 1.0 μl of each primer (10 pmol/μl), 0.1 μl Taq DNA polymerase (5 U/μl; Bioline) and 1.0 μl genomic DNA (100 ng/μl). Reference strains 125.91 (= CBS10512; **a**A; AFLP1), H99 (= CBS8710; αA; AFLP1), JEC20 (= CBS10511; **a**D; AFLP2) and JEC21 (= CBS10513; αD; AFLP2) were included as controls.

Amplified Fragment Length Polymorphism (AFLP) fingerprinting was performed to genotype the strains as previously described (Barreto de Oliveira *et al.*, 2004; Boekhout *et al.*, 2001). The reference strains H99 (= CBS8710; AFLP1), Bt63 (= CBS11550; AFLP1A), WM626 (=CBS10083; AFLP1B), JEC21 (= CBS10513; AFLP2), CBS132 (AFLP3), WM179 (= CBS10078; AFLP4), WM161 (= CBS10081; AFLP5), WM178 (= CBS10082; AFLP6), WM779 (= CBS10101; AFLP7), CBS10488 (AFLP8), CBS10496 (AFLP9) and CBS11687 (AFLP10) were included to assign the AFLP genotypes to each of the clinical strains (Hagen *et al.*, 2010; Meyer *et al.*, 2009).

Statistical analysis of results

The MIC_{50}, MIC_{90} and geometric mean MICs of each antifungal agent were calculated. Only the first strain for every patient was used in the analysis of relationship between *in vitro* susceptibility for five tested antifungal compounds and the patient's immune status and the different mating-types, serotypes, and genotypes of *C. neoformans* strains. To compare two groups the Mann-Whitney test was applied, while the Kruskal-Wallis test was used to compare more than two groups. Statistical analyses were performed using SPSS v12 (SPSS, Chicago, IL), statistical significance was determined when a P of ≤0.05 was observed.

Results

Mating-type, serotype and AFLP genotype analyses

PCR-based mating-type and serotype determination revealed that cryptococcosis in the majority of patients was caused by mating-type α (*n*=12; 80.0%) while the remaining three (20.0%) patients were found to be infected by cryptococcal strains with both mating-types α and **a** (Table 1). Combined with the serotype of these strains, it was found that six (40.0%) patients were infected with αA, another six (40.0%) with αD, two (13.3%) with αA**a**D and one (6.7%) patient with αD**a**A. The frequency of cryptococcosis caused by genotype AFLP1 and AFLP2 strains was for both six (40.0%), genotype AFLP3 strains were observed to have infected three patients (20.0%).

AFLP fingerprinting revealed more diversity among strains that were isolated from the same patient (Figure 1). No discrepancies between the determined mating- and serotyping results and the AFLP genotyping were observed.

None of the patients were found to be infected with *C. neoformans* strains that had different mating-types or serotypes (Figure 1; Table 1). However, the AFLP fingerprint patterns showed that there were genotypic differences among strains from the same patient. The majority of differences between 'same patient strains' were found to be minor changes in the AFLP fingerprint patterns, thus absence or presence of minor fragments (Figure 1). It was observed that patient 8 was infected by two different *C. neoformans* genotypes. The first two *C. neoformans* var. *grubii* strains cultured from patient 8 were found to be genotypic similar, namely AFLP1B, while the third strains from day eleven was found to belong to genotype AFLP1 (Figure 1). Two out of eleven *C. neoformans* var. *neoformans* strains from patient 7, isolated on day one and two of hospitalisation, were found to be genotypically slightly different from the other strains that were isolated upon the following 56 days, as is indicated by the absence of some major fragments (Figure 1). However, the *C. neoformans* var. *neoformans* strain cultured on the first two days remained persistent since a similar genotypic strain was found in the CSF sample taken on day 39. Similar observations were made for the *C. neoformans* strains cultured from patient 1 and 6. Six strains were available from patient 1, the strains from the first day of hospitalisation was found to be similar to those isolated four and eleven days after the first sample was taken. Genotypically slightly different strains were cultured during the three days following the first sample. For patient 6, the strain on the first day of hospitalisation had additional fragments in its AFLP fingerprint pattern compared to strains cultured during successive days of isolation. Interestingly, the absence of two minor fragments in the AFLP fingerprint profile of the *C. neoformans* genotype AFLP3 strains CRO08 and CRO09 cultured from patient 3 caused an apparent problem for the AFLP fingerprint clustering software since these strains were clustered in different clades (Figure 1).

Next page, Figure 1. AFLP fingerprint patterns of 48 Croatian *C. neoformans* strains. AFLP genotype, mating- and serotype, patient number, source of isolation and HIV-status of the patient as well as the day of isolation during hospitalisation are indicated after the AFLP fingerprint pattern. Examples of differences in AFLP fingerprint patterns among sequentially strains of *C. neoformans* are indicated by a box. Three strains that were isolated from patient 8 are indicated by asterisks to highlight the different AFLP genotypes that these strains exhibit.

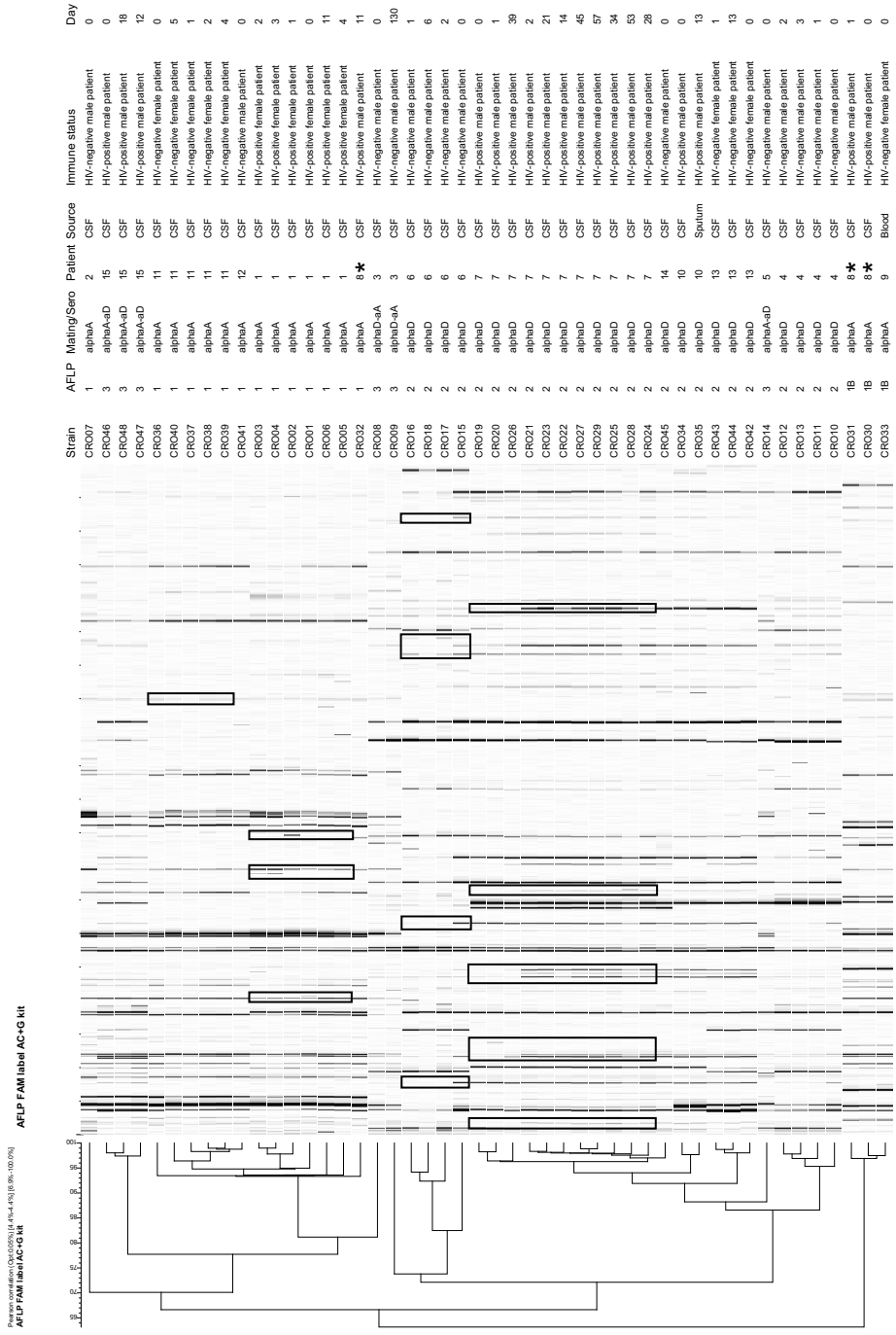
Strain AFLP Mating/Sero Patient Source Immune status Day
CRO07 1 alphaA 2 CSF HIV-negative male patient 0
CRO46 3 alphaA-aD 15 CSF HIV-positive male patient 0
CRO48 3 alphaA-aD 15 CSF HIV-positive male patient 18
CRO47 3 alphaA-aD 15 CSF HIV-positive male patient 12
CRO36 1 alphaA 11 CSF HIV-negative female patient 0
CRO40 1 alphaA 11 CSF HIV-negative female patient 5
CRO37 1 alphaA 11 CSF HIV-negative female patient 1
CRO38 1 alphaA 11 CSF HIV-negative female patient 2
CRO39 1 alphaA 11 CSF HIV-negative female patient 4
CRO41 1 alphaA 12 CSF HIV-negative male patient 0
CRO03 1 alphaA 1 CSF HIV-positive female patient 2
CRO04 1 alphaA 1 CSF HIV-positive female patient 3
CRO02 1 alphaA 1 CSF HIV-positive female patient 1
CRO01 1 alphaA 1 CSF HIV-positive female patient 0
CRO06 1 alphaA 1 CSF HIV-positive female patient 11
CRO05 1 alphaA 1 CSF HIV-positive female patient 4
CRO32 1 alphaA 8* CSF HIV-positive male patient 11
CRO08 3 alphaD-aA 3 CSF HIV-negative male patient 0
CRO09 3 alphaD-aA 3 CSF HIV-negative male patient 130
CRO16 2 alphaD 6 CSF HIV-negative male patient 1
CRO18 2 alphaD 6 CSF HIV-negative male patient 6
CRO17 2 alphaD 6 CSF HIV-negative male patient 2
CRO15 2 alphaD 6 CSF HIV-negative male patient 0
CRO19 2 alphaD 7 CSF HIV-positive male patient 0
CRO20 2 alphaD 7 CSF HIV-positive male patient 1
CRO26 2 alphaD 7 CSF HIV-positive male patient 39
CRO21 2 alphaD 7 CSF HIV-positive male patient 2
CRO23 2 alphaD 7 CSF HIV-positive male patient 21
CRO22 2 alphaD 7 CSF HIV-positive male patient 14
CRO27 2 alphaD 7 CSF HIV-positive male patient 45
CRO29 2 alphaD 7 CSF HIV-positive male patient 57
CRO25 2 alphaD 7 CSF HIV-positive male patient 34
CRO28 2 alphaD 7 CSF HIV-positive male patient 53
CRO24 2 alphaD 7 CSF HIV-positive male patient 28
CRO45 2 alphaD 14 CSF HIV-negative male patient 0
CRO34 2 alphaD 10 CSF HIV-positive male patient 0
CRO35 2 alphaD 10 Sputum HIV-positive male patient 13
CRO43 2 alphaD 13 CSF HIV-negative female patient 1
CRO44 2 alphaD 13 CSF HIV-negative female patient 13
CRO42 2 alphaD 13 CSF HIV-negative female patient 0
CRO14 3 alphaA-aD 5 CSF HIV-negative male patient 0
CRO12 2 alphaD 4 CSF HIV-negative male patient 2
CRO13 2 alphaD 4 CSF HIV-negative male patient 3
CRO11 2 alphaD 4 CSF HIV-negative male patient 1
CRO10 2 alphaD 4 CSF HIV-negative male patient 0
CRO31 1B alphaA 8* CSF HIV-positive male patient 1
CRO30 1B alphaA 8* CSF HIV-positive male patient 0
CRO33 1B alphaA 9 Blood HIV-negative female patient 0
Pearson correlation (Ward) 95% [14.4%-4.4%] [8.8%-100.0%]
AFLP FAM label AC+G kit
AFLP FAM label AC+G kit
100
95
90
85
80
75
70
65

Table 2. MIC ranges, geometric mean MICs, MIC_{50}s, MIC_{90}s and percentages of *C. neoformans* strains per MIC group.

	MIC values (µg/ml) of *Cryptococcus* strains				**Percentages of *Cryptococcus* strains per MIC group (µg/ml)**							
	Range	**MIC_{50}**	**Geometric Mean MIC**	**MIC_{90}**	**0.031**	**0.062**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
Amphotericin B	0.031 - 0.5	0.062	0.100	0.5	2.1 %	52.1%	27.1%	6.2%	12.5%	-	-	-
5-Flucytosine	1 - 4	4	2.788	4	-	-	-	-	-	18.7%	14.6%	66.7%
Fluconazole	1 - 4	2	1.658	4	-	-	-	-	-	43.7%	39.6%	16.7%
Itraconazole	0.031 - 0.5	0.125	0.113	0.25	6.2%	25.0%	52.1%	10.4%	6.2%	-	-	-
Voriconazole	0.031 - 0.25	0.062	0.059	0.062	16.7%	75.0%	6.2%	2.1%	-	-	-	-

Table 3. Table 3. Relationship between *in vitro* susceptibility for five antifungal compounds and patients immune status and the different mating-types, serotypes and genotypes of *C. neoformans*.

	Patients immune status			**Strain mating-type**			**Strain serotype / AFLP genotype**			
Category	**HIV positive**	**HIV negative**	***P*-value**	**α**	**α/a**	***P*-value**	**A / AFLP1**	**D / AFLP2**	**AD / AFLP3**	***P*-value**
$n_{patients}$	5	10	-	12	3	-	6	6	3	-
Amphotericin B	0.062	0.100	0.242	0.091	0.062	0.624	0.111	0.076	0.062	0.866
5-Flucytosine	2.000	3.250	0.159	2.670	3.170	0.795	2.240	3.170	3.170	0.516
Fluconazole	1.320	1.870	0.346	1.590	2.000	0.533	2.000	1.260	2.000	0.311
Itraconazole	0.082	0.134	0.294	0.118	0.099	0.879	0.198	0.070	0.098	0.079
Voriconazole	0.071	0.044	0.051	0.047	0.078	0.067	0.044	0.049	0.078	0.163

Antifungal susceptibility testing

Detailed results of the broth microdilution antifungal susceptibility testing for all 48 clinical strains have been summarized in Table 1 and 2. All five antifungal compounds demonstrated high *in vitro* activity against all *C. neoformans* strains. The MIC_{90} for amphotericin B was 0.5 μg/ml, for 5-flucytosine and fluconazole 4 μg/ml, itraconazole 0.25 μg/ml and for voriconazole 0.062 μg/ml. The MIC range for amphotericin B was 0.031 - 0.5 μg/ml, MIC ranges for 5-flucytosine and fluconazole were identical (1 - 4 μg/ml), while the MIC range of itraconazole was found to be broader (0.031 - 0.5 μg/ml) than that of voriconazole (0.031 - 0.25 μg/ml).

No significant differences in susceptibility to any of the five tested antifungal compounds were observed when the patients HIV–status, or the mating-type, serotype or AFLP genotype of the *C. neoformans* strains were analysed (Table 3).

Discussion

The present retrospective study describes the epidemiology and *in vitro* antifungal susceptibility profiles of sequentially straind *Cryptococcus neoformans* strains from fifteen Croatian patients during a five-year period (2005-2010). This study was initiated due to the absence of any epidemiological data regarding *Cryptococcus* infections in Eastern Europe.

In vitro *antifungal susceptibility profiles*

The tested antifungal compounds amphotericin B, 5-flucytosine, fluconazole, itraconazole and voriconazole demonstrated high *in vitro* activity against *C. neoformans* strains (Table 1). The highest geometric mean MIC was found for 5-flucytosine (2.790 μg/ml), and the lowest for voriconazole (0.060 μg/ml) (Table 2). Despite the increase in *in vitro* MIC values by one to three $\log_2$ dilutions, the maximum MIC values for these sequential strains obtained during the course of a single or multiple episodes of cryptococcosis remained *in vitro* susceptible for all tested antifungal agents (Table 1).

Similarly with the clinical strains of *C. neoformans* from Croatia, large percentages (~99%) of tested clinical strains from Africa, Europe, North America, South America and the Pacific Region (Pfaller *et al.*, 2005), Cuba (Illnait-Zaragozí *et al.*, 2008), France (Dannaoui *et al.*, 2005), Spain (Guinea *et al.*, 2010; Lopez-Jondra *et al.*, 2000), Brazil (Fusco Almeida *et al.*, 2007; Souza *et al.*, 2005) and the USA (Brandt *et al.*, 2001) proved to be *in vitro* susceptible (MIC ≤1 μg/ml) to amphotericin B. However, detection of *in vitro* resistance to amphotericin B (MIC ≥2 μg/ml) in 5.3% of the studied clinical *C. neoformans* strains from a Spanish collection seems to be different from the current study as well as from the other aforementioned studies (Perkins *et al.*, 2005).

In concordance with the results of the current study, previous studies have also found *in vitro* susceptibility of all investigated clinical *C. neoformans* strains to itraconazole (MIC ≤0.5 μg/ml) (Datta *et al.*, 2003; Lopez-Jondra *et al.*, 2000; Souza *et al.*, 2005) and voriconazole (MIC ≤0.5 μg/ml) (Guinea *et al.*, 2010; Illnait-Zaragozí *et al.*, 2008; Perkins *et al.*, 2005; Souza *et al.*, 2005). Exceptions were the *in vitro* resistance

(MIC ≥1 μg/ml) to itraconazole for less than ten percent of the studied *C. neoformans* strains (Brandt *et al*., 2001; Fusco Almeida *et al*., 2007; Illnait-Zaragozí *et al*., 2008) and even up to 15.8% for a group of Spanish *C. neoformans* strains (Perkins *et al*., 2005).

Results of the *in vitro* susceptibility against fluconazole and 5-flucytosine for the Croatian clinical *C. neoformans* strains are in concordance with other recent studies. For fluconazole we observed a MICs <16 μg/ml which is similar to data given by Dannaoui *et al*. (2006), Fusco Almeida *et al*. (2007) and Souza *et al*., (2005), while the MIC of <32 μg/ml for 5-flucytosine was similar to those found by Dannaoui *et al*. (2006) and Lopez-Jondra *et al*. (2000). On the contrary to this, *in vitro* resistance to fluconazole (MIC ≥16 μg/ml) was proven in 3.4% of *C. neoformans* strains originating from Spain (Guinea *et al*., 2010), 15.9% from India (Datta *et al*., 2003), 19% from the USA (Brandt *et al*., 2001), 25% from North America (Pfaller *et al*., 2005), and 46.6% from Spain (Perkins *et al*., 2005). The percentage of clinical *C. neoformans* strains resistant to 5-flucytosine (MIC ≥32 μg/ml) was lower and also depending upon geographical area. While all Croatian *C. neoformans* strains were found to be susceptible for 5-flucytosine, 1% of the African, European, Pacific and North and South American strains (Pfaller *et al*., 2005), 3.8% of the strains from the USA (Brandt *et al*., 2001) and 4.7% of the studied strains from Spain (Perkins *et al*., 2005) were observed to be resistant.

The difference in *in vitro* antifungal susceptibility between the different genotypes and mating-types within *C. neoformans* has not been studied extensively. To our best knowledge, the current study is the second known next to that of Guinea *et al*. (2010). The current study (Table 3) and that of Guinea *et al*. (2010) have observed that the genotypic background of a cryptococcal strain (genotype AFLP 1-3) or the mating-type α, has no statistically significant influence on the *in vitro* antifungal activity against fluconazole and voriconazole. On the contrary to the current results (Table 3), the Spanish epidemiological study (Guinea *et al*., 2010) observed a statistically significant correlation of *in vitro* susceptibility to amphotericin B in relation to the AFLP genotypes of the *C. neoformans* strains.

Several studies have evaluated the relationship between antifungal susceptibility and serotypes of *C. neoformans* strains. There was no significant difference observed between the antifungal susceptibility values from the five antifungal compounds when compared to the serotype A, D and AD background of the strains (Table 3). The French (Dannaoui *et al.,* 2006) and Italian study (Tortorano *et al.,* 1997) showed that serotype A strains were significantly less susceptible to fluconazole than serotype D. However, this discrepancy between serotype A and D was not observed in the Spanish study (Guinea *et al.*, 2010). The French (Dannaoui *et al.*, 2006) and the Spanish studies (Guinea *et al.*, 2010) observed that *in vitro* antifungal susceptibility to amphotericin B was lower in serotype A than in serotype D strains. The French *C. neoformans* serotype A strains were also less susceptible to 5-flucytosine than serotype D strains (Dannaoui *et al.,* 2006). In concordance with the results of the current study (Table 3), the susceptibility against fluconazole and voriconazole for different serotypes of *C. neoformans* was similar to those that were observed in the Spanish study (Guinea *et al.*, 2010).

Sequential C. neoformans *strains from the same patient*

A handful of studies are published that describe the occurrence of mixed *C. neoformans* infections or shifts in genotypic and antifungal susceptibility differences among sequentially straind *C. neoformans* strains (Blasi *et al.*, 2001; Casadevall *et al.*, 1993; Desnos-Ollivier *et al.*, 2010; Illnait Zaragozi *et al.*, 2010; Pfaller *et al.*, 1998; Spitzer *et al.*, 1993; Sullivan *et al.*, 1996). It has been shown that genomic differences between sequential strains of *C. neoformans* can influence virulence and persistence of a cryptococcal infection, as has been shown using a mice pathogenicity model (Fries & Casadevall, 1998). Besides a change in the genotype of the initial *C. neoformans* strain due to microevolution or karyotype instability, replacement of the initial strain with another genotypic variant can influence the outcome of antifungal treatment or may cause a relapse (Blasi *et al.*, 2001; Fries & Casadevall, 1998).

From the patients from whom we have studied multiple *C. neoformans* strains, only patient 8 was found to be infected with multiple genotypic *C. neoformans* variants, namely genotype AFLP1 and AFLP1B strains (Figure 1; indicated by asterisks). Several other patients (1, 6 and 7) were found to be infected with strains that show minor differences in the AFLP fingerprint patterns. Interestingly, it was observed that the eleven *C. neoformans* var. *neoformans* strains from patient 7 became gradually less susceptible when tested *in vitro* against amphotericin B (0.062 to 0.5 µg/ml), 5-flucytosine (1 to 4 µg/ml), fluconazole (1 to 2 µg/ml) and itraconazole (0.031 to 0.125 µg/ml), while no change in voriconazole susceptibility was observed (Table 1). The gradual decrease in *in vitro* antifungal susceptibility of these strains might be due to microevolution that took place in the *C. neoformans* strains during the course of antifungal therapy that this patient underwent. Patient 7 was initially treated with colloidal amphotericin B (400 mg/d iv) and fluconazole (400 mg/d iv). After three weeks of therapy the dosage of fluconazole was doubled. Different formulations of amphotericin B were continuously given for a total of two months, after which high dosage of fluconazole (1600 mg/d iv) was administrated. HIV-infection in patient 7 was confirmed after the diagnosis of cryptococcal meningitis. The patient had a $CD4^+$ cell count of 25 cells per mm^3 and a plasma RNA HIV-1 viral load of 956,000 copies per ml. HAART, with stavudine, lamivudine and nevirapine, was given after three weeks of antifungal therapy. After six weeks of HAART, the $CD4^+$ cell count increased to 96 cells per mm^3, and the plasma viral load decreased to 1,490 HIV-1 RNA copies per ml. However, the patient did not recover and died three months after the diagnosis of cryptococcal meningitis was made. The HIV-infected patient 7 showed to be initially infected with a *C. neoformans* var. *neoformans* strain that was replaced by another genotypic variant after the second day while the initial genotypic variant was again straind on day 39 (Figure 1; Table 1).

The case of patient 7 is comparable to that observed by Spitzer *et al.* (1993) who showed that initial and recurrent *C. neoformans* strains were clonally related. This confirms that an initial *C. neoformans* strain can persist despite antifungal therapy rather than that a novel infection is acquired. Thus therapeutic failure might occur due to the fact that an antifungal treatment strategy cannot eradicate the initial infection (Spitzer *et al.*, 1993). On the contrary, it has also been observed that sequentially *C. neoformans* strains became *in vitro* increasingly susceptible for fluconazole (Casadevall

et al., 1993). This provides evidence against antifungal resistance as the cause of recurrent *C. neoformans* infection, and it might be that an attenuated immune system can give rise to the persistence of cryptococcal infections (Casadevall *et al.*, 1993).

For the *C. neoformans* cultures from patient 8, higher MIC values were found between two initial and the last strain for amphotericin B (0.062 to 0.125 µg/ml) (Table 1). This patient was treated initially with colloidal amphotericin B (400 mg/d iv) plus fluconazole (800 mg/d iv). After five days, colloidal amphotericin B was replaced by liposomal amphotericin B (300 mg/d iv) and the dose of fluconazole was lowered (400 mg/d iv). Five days later, the treatment was continued with amphotericin B deoxycholate (75 mg/d iv) and the same dosage of fluconazole (400 mg/d iv). Different formulations of amphotericin B were given for a total of 3 weeks. Fluconazole (400 mg/d orally), as maintenance therapy, was administered for 12 months. At follow up, the patient was fully recovered.

Mixed infections have been recently in detail described by Desnos-Ollivier *et al.* (2010) who observed that nearly twenty percent of the investigated patients were infected with multiple *C. neoformans* genotypic variants. They also found that serotype A and D strains can form serotype AD hybrids *in vivo* using a mice model. Thus it is likely that *C. neoformans* is able to form *in vivo* new genotypic variants that eventually might be less susceptible against antifungal compounds. In the current study, patient 8 was found to be initially infected with an strain belonging to genotype AFLP1B while ten days later a genotypically different strain with genotype AFLP1 was cultured. Despite the fact that only one *C. neoformans* colony was used for further characterisation, the possibility exist that the initial *C. neoformans* AFLP1B strain underwent genotypic changes to an AFLP1 genotype due to the selection pressure caused by the antifungal treatment. This might be a plausible explanation since it has been hypothesized that *C. neoformans* genotype AFLP1A and AFLP1B strains might be hybrid genotypes (Barreto de Oliveira *et al.*, 2004; Boekhout *et al.*, 2001). Alternatively, the patient could be infected with multiple *C. neoformans* genotypic variants but due to the fact that only one colony was investigated per sample, an initial mixed infection might be overlooked. This explanation has also been given for a similar observation for sequentially strains of *C. neoformans* from Cuban patients, four out of nineteen patients were found to be probably infected by more than one *C. neoformans* genotypic variant but only a single colony was investigated (Illnait-Zaragozí *et al.*, 2010).

In conclusion, no *in vitro* resistance to amphotericin B, 5-flucytosine, fluconazole, itraconazole, and voriconazole was observed among the 48 clinical *C. neoformans* strains from Croatia that were collected between 2005 and 2010. A statistically significant correlation was not demonstrated between *in vitro* antifungal susceptibility patterns and HIV status of the patients, or the mating-, serotype and AFLP genotype of the studied *C. neoformans* strains. Multiple *C. neoformans* genotypic variants might exist during the initial infection and therefore it is recommended to investigate multiple single colonies during the course of infection when a patient is not responding on the antifungal treatment.

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Chapter 5

Emergence of autochthonous and dormant *Cryptococcus gattii* infections in Europe: a Multi-Locus Sequence Typing approach

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Submitted.

Summary

During the past decade the basidiomycetous yeast *Cryptococcus gattii* has stepped out of the shadows of it sibling, the opportunistic pathogen *C. neoformans*. Until a decade ago, *C. gattii* infections were mainly seen in tropical and subtropical climate zones, but the sudden onset of the *C. gattii* outbreak at Vancouver Island (British Columbia, Canada) and its recent expansion to the mainland of Canada and the Pacific Northwest has shown that it can survive in more temperate climates. In Europe, *C. gattii* is found to cause infections in humans and animals with an increased frequency. To determine if European *C. gattii* cases were acquired during travelling to endemic regions or that they originated from an autochthonous source, a set of 107 European isolates was compared to 194 globally collected isolates. The genetic relatedness of these isolates was studied using Multi-Locus Sequence Typing based on ten nuclear loci. This revealed that environmental, veterinary and clinical isolates from Mediterranean Europe fell into a distinct *C. gattii* genotype. Clinical isolates from Northwestern Europe could be linked to environmental isolates from other continents indicating that reactivation of dormant *C. gattii* infections may occur many years after acquiring the infection.

Introduction

During the past decade the basidiomycetous yeast *Cryptococcus gattii* has stepped out of the shadows of it sibling *C. neoformans*. The latter is known to cause cryptococcosis mainly in immunocompromised patients, while *C. gattii* primarily infects apparently immunocompetent individuals. The increase of cryptococcosis started during the onset of the HIV/AIDS pandemic in the early 1980s, and before that period cryptococcosis was relatively a rare cause of disease. It has been estimated that every year nearly a million HIV-infected individuals develop cryptococcal meningitis, mainly in sub-Saharan Africa, and approximately 65% of these patients die due to this infection (Park *et al.*, 2009). On the contrary to *C. neoformans* that occurs globally, *C. gattii* was previously found to be geographically restricted to tropical and subtropical localities in Central Africa, Northern Australia and Central and South America (Kwon-Chung & Bennett, 1984; Springer & Chaturvedi, 2010). However, this distribution pattern changed by an unprecedented outbreak of *C. gattii* which emerged at the end of the 1990s in the temperate climate of British Columbia (Canada) and that recently expanded to the Pacific Northwest (Datta *et al.*, 2009). Recent retrospective epidemiological studies have shown that *C. gattii* outbreaks occur in other temperature climate zones, such as in Mediterranean Europe and Western Australia (Baró *et al.*, 1998; Carriconde *et al.*, 2011; Colom *et al.*, 2011).

Cryptococcus gattii was recognized as a species a decade ago, and before that time it was known as a variety of *C. neoformans* as *C. neoformans* var. *gattii* (Kwon-Chung *et al.*, 2002). Morphological differences between *C. neoformans* and *C. gattii* become visible when the species enters the sexual phase. The teleomorphic phases were named *Filobasidiella neoformans* and *F. bacillispora*, respectively (Kwon-Chung, 1975, 1976). In the past serotyping was frequently used to differentiate *C. neoformans* from *C. gattii*, but this test is no longer commercially available. Using the agglutination serotyping technique it is possible to discern four different serotypes (Ikeda *et al.*, 1982). Serotype A and D represents *C. neoformans* var. *grubii* and var. *neoformans*, respectively, and serotype B and C representing *C. gattii* (Kwon-Chung *et al.*, 1982). Recently, it was found that besides the intervarietal 'serotype AD hybrid', also interspecies hybrids can be formed which were named 'serotype AB' and 'serotype BD' hybrids (Bovers *et al.*, 2006, 2008a, 2008c). Besides these differences, both species can phenotypically be separated using L-canavanine-glycine-bromothymol blue medium (Klein *et al.*, 2009; Kwon-Chung *et al.*, 1982).

A broad range of molecular biological techniques have been developed to characterize cryptococcal isolates, including PCR fingerprinting, Restriction Fragment Length Polymorphism (RFLP) analysis of the *PLB1* and *URA5* loci, Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis, and several Multi-Locus Sequence Typing (MLST) approaches (Boekhout *et al.*, 2001; Bovers *et al.*, 2008a, 2008b; Meyer *et al.*, 2009). These approaches showed that *C. gattii* can be divided into five distinct genotypes, named AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV, and AFLP10/VGIV (Hagen *et al.*, 2010a; Meyer *et al.*, 2009). Serotype B strains occur in genotype AFLP4/VGI, AFLP6/VGII and AFLP10/VGIV, while serotype C strains are restricted to

genotypes AFLP5/VGIII and AFLP7/VGIV (Hagen *et al.*, 2010a).

Multi-Locus Sequence Typing (MLST) has become a widely used typing method to investigate the population structure of pathogenic micro-organisms and has especially been applied in studies on prokaryote pathogens and to a lesser extent for fungal pathogens (Jacobsen *et al.*, 2008; Klaassen, 2009; Maiden, 2006). MLST analysis has been applied to study the taxonomy, epidemiology and population structure of several human fungal pathogens, including *Cryptococcus* (Bovers *et al.*, 2008b; Carriconde *et al.*, 2011; Fraser *et al.*, 2005; Kidd *et al.*, 2005; Litvintseva *et al.*, 2006; Ngamskulrungroj *et al.*, 2009; Simwami *et al.*, 2011). However, each of these studies used different nuclear loci and different numbers of loci. Therefore, a consensus MLST scheme has been proposed consisting of the seven nuclear loci *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5* (Meyer *et al.*, 2009). So far, this consensus MLST scheme has been used to study the population structure of Thai *C. neoformans* and Australian *C. gattii* strains (Carriconde *et al.*, 2011; Simwami *et al.*, 2011).

The current study was initiated to investigate the occurrence, apparent emergence and epidemiology of *C. gattii* in Europe among clinical, veterinary and environmental sources. This will provide a better understanding of the genetic diversity of this primary pathogen in Europe and will provide a base to study the link between disease, geographical region and, if possible, where infections were acquired. AFLP fingerprint typing was initially applied to genotype the isolates, while MLST was used to assess the genetic diversity. To achieve this, previously published *C. gattii* MLST studies by Fraser *et al.* (2005) and Byrnes *et al.* (2010) were used and extended to match with the *Cryptococcus* consensus MLST scheme (Meyer *et al.*, 2009). Our studies revealed that i) all *C. gattii* genotypes occur in Europe, ii) genotype AFLP4/VGI isolates predominate, iii) a Mediterranean *C. gattii* cluster exist that is genetically distinct from the other populations and may be autochthonous and iv) several clinical cases were caused due to travel related *C. gattii* infections.

Material and Methods

Isolates and media

A set of 107 (35.5%) European isolates were compared to a set of 194 globally collected isolates with an African (*n*=36; 12.0%), Asian (*n*=26; 8.6%), Australian (*n*=18; 6.0%), North American (*n*=71; 23.6%), South American (*n*=38; 12.6%), or unknown geographic origin (*n*=5; 1.7%). The source of these isolates were from clinical (*n*=190; 63.1%), veterinary (*n*=48; 16.0%), environmental (*n*=59; 19.6%) and unknown sources (*n*=4; 1.3%). A detailed overview of the geographic origin and sources of these *C. gattii* isolates is provided in Table 1.

The full data of all *C. gattii* isolates is provided in Supplementary Table 1. All isolates were checked for purity and cultivated on Malt Extract Agar medium (MEA; Oxoid, Basingstoke, United Kingdom). Cultures were incubated for two days at 30°C. A working collection was made by growing *C. gattii* isolates on MEA slants for two days at 30°C after which they were stored at 4°C. Furthermore, isolates were stored at -80°C using the Microbank™ system (Pro-Lab Diagnostics, Richmond Hill, ON).

Genomic DNA extraction, AFLP fingerprinting and mating-type determination
Genomic DNA was extracted as described before by Hagen *et al.* (2010a) and working solutions with a concentration of 100 ng/µl were prepared using Tris-EDTA (pH 8.0). Genotypes of the *C. gattii* isolates were determined by Amplified Fragment Length Polymorphism (AFLP) fingerprinting as previously described (Boekhout *et al.*, 2001; Hagen *et al.*, 2010a). The *C. gattii* isolates WM178 (= CBS10082; AFLP6/VGII), A1M-R265 (= CBS10514; AFLP6A/VGIIa), A1M-R272 (= CBS10865; AFLP6B/VGIIb), A6M-R38 (= CBS11545; AFLP6C/VGIIc), WM161 (= CBS10081; AFLP5/VGIII), WM179 (= CBS10078; AFLP4/VGI), WM779 (= CBS10101; AFLP7/VGIV), CBS10488 (AFLP8), CBS10496 (AFLP9) and IHEM14941S (= CBS11687; AFLP10) were used as controls to assign the AFLP genotypes to all *C. gattii* isolates (Hagen *et al.*, 2010a; Meyer *et al.*, 2009).

The mating-types of the *C. gattii* isolates were determined by amplification of the *STE12***a** or the *STE12*α locus as previously described (Hagen *et al.*, 2010a). As a control, *C. gattii* mating-type α strains CBS6956 (AFLP6A/VGIIa) and WM276 (= CBS10510; AFLP4/VGI) and mating-type **a** strains CBS1930 (AFLP6B/VGIIb) and E566 (= CBS11233; AFLP4/VGI) were included.

Amplification and sequencing of Multi-Locus Sequence Typing loci
According to a recently established consensus Multi-Locus Sequence Typing (MLST) scheme, the seven nuclear loci *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5* were amplified using the preferred primer combinations (Meyer *et al.*, 2009). To compare the current set of *C. gattii* isolates with that of a previously published *C. gattii* population study, the three nuclear loci *CAP10*, *MPD1* and *TEF1*α (Fraser *et al.*, 2005) that are not part of the consensus MLST scheme were included in our study as well. Additionally, available isolates that were part of a global collection and that were used in previous studies of Fraser *et al.* (2005) and Byrnes *et al.* (2010) were included and subjected to amplification and sequencing of the missing nuclear loci *CAP59*, *SOD1* and *URA5*. New primer pairs for the loci *CAP10*, *CAP59*, *GPD1*, *MPD1* and *TEF1*α were developed since the original primer combinations were empirically found to be too specific for certain *C. gattii* genotypes. The newly used forward and reversed primer sequences were 5'-GTCGTTTTCGCCGATCCTC-3' and 5'-GCCGTAAGACGTGCCCA-3' for *CAP10*, 5'-GTGAACAAGCTGCGGC-3' and 5'-GGATTCAGTGTGGTGGAAGA-3' for *CAP59*, 5'-GGTTGTCAAGGTTGGAATCAACGG-3'and'5-GGAGCGGAAATGACGACCTTCTT-3'for *GPD1*, '5- CCCAGACTGCCGCTGT-3' and '5- GTGCCGCTAGGCTTCAAGTA-3' for *MPD1* and '5-CTCGGACGGCGAATCGACCAAGAGG-3' and '5- GACGGTCAGACCCGAGAGCA CGC-3'for *TEF1*α.

Amplifications were carried out in a 25 µl PCR mixture consisting of 17.8 µl ddH_2O, 0.75 µl $MgCl_2$ (50 mM; Bioline, London, United Kingdom), 2.5 µl 10× PCR Buffer (Bioline), 1.9 µl dNTP's (1 mM; Bioline),0.1 µl Taq DNA polymerase (5 U/µl; Bioline), 0.5 µl of the forward and reversed primer (10 pmol/µl; Biolegio, Nijmegen, The Netherlands) and 1 µl genomic DNA (100 ng/µl). PCR reactions were carried out with an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds (60°C for *CAP10*, 58°C for *CAP59*, 61°C for *GPD1*, 61°C for IGS1, 50°C for *LAC1*, 51°C for *MPD1*, 56°C for *PLB1*, 52°C for *SOD1*, 62°C

for *TEF1α* and 63°C for *URA5*) and extension at 72°C for 1 minute, followed by 72°C for 5 minutes and a final dwell of 21°C. Amplification reactions were checked by agarose gel electrophoresis and reactions that were found to be too concentrated for sequencing were diluted with ddH_2O prior further processing.

Sequencing reactions were carried out with the BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) as described by Bovers *et al.* (2008b). For all amplification products, except *CAP59*, the initial amplification primers were used for sequencing reactions. For *CAP59*, the newly designed forward primer CAP59L-Forward and the reversed primer JOHE15438 (Fraser *et al.*, 2005) were used. Prior to analysis on an ABI 3700XL DNA Analyzer (Applied Biosystems), the sequencing reactions were purified with Sephadex G-50 Superfine columns (GE Healthcare, Waukesha, WI) and a Multi-Screen HV plate (Millipore, Billerica, MA).

Sequence alignments, phylogenetic and recombination analyses

Consensus sequences were assembled and checked for ambiguities using SeqMan v8.0.2 (DNASTAR, Madison, WI). Sequence alignments per locus were generated with MEGA v5 (Tamura *et al.*, 2011) using the standard settings and manual correction. The genome sequence databases of reference strains H99 (= CBS8710; *C. neoformans* var. *grubii*; AFLP1; Broad Institute, http://www.broadinstitute.org/) and JEC21 (= CBS10513; *C. neoformans* var. *neoformans*; AFLP2; Stanford Genome Technology Center, http://www-sequence.stanford.edu/) were used to extract the corresponding sequences for all ten investigated nuclear loci to serve as outgroup. The best fitting nucleotide substitution model was determined using MrModeltest v2 (Nylander, 2004). This was done for the complete *C. gattii* ten-loci MLST as well as for the accepted consensus MLST scheme (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5*; Meyer *et al.*, 2009) and a previously launched *C. gattii* MLST scheme (*CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1*, *TEF1α*; Fraser *et al.*, 2005). As a result, the Hasegawa-Kishino-Yano plus gamma distributed with invariant sites model (HKY G+I) was the best model to analyze the phylogeny of the *C. gattii* isolates for all three datasets. The evolutionary history was inferred using the Maximum Likelihood (ML) method conducted in MEGA v5 (Tamura *et al.*, 2011). A bootstrap consensus tree was inferred from 1000 replicates to show the significant lineages obtained in this analysis.

The haplotype diversity (H_R), equal to the Simpsons diversity index (D), was calculated using the Microsoft Excel add-in 'Haplotype Analysis' (Eliades & Eliades, 2009; Microsoft, Redmond, WA). For this purpose, sequences were collapsed into sequence type numbers (STs) since this software tool was designed to handle numerical data (Supplementary Table 1). Recombination analysis was performed with clone corrected sequence data using the pairwise homoplasy index (Φ_w) method implemented in SplitsTree v4 (Bruen *et al.*, 2006; Huson & Bryant, 2006).

Results

AFLP genotyping and geographic distribution of all studied* C. gattii *isolates

AFLP genotyping revealed that the 301 *Cryptococcus gattii* isolates could be divided into 146 AFLP4/VGI (48.5%), 22 AFLP5/VGIII (7.3%), 108 AFLP6/VGII (35.9%), 13 AFLP7/VGIV (4.3%), two AFLP10/VGIV (0.7%), seven AFLP8 (2.3%) and three AFLP9 (1.0%) isolates. Ten isolates (3.3%) exhibited genotype AFLP8 and AFLP9, representing the interspecies *C. neoformans* × *C. gattii* hybrids and these were excluded for further MLST analysis due the fact that amplified fragments of hybrid isolates will result in mixtures of different alleles.

The genotypic diversity of the remaining 291 *C. gattii* isolates with a haploid genotype is listed in Table 1 and categorized per continent and per category of clinical, veterinary, environmental or unknown sources. The most frequently observed genotypes in each continental population were AFLP4/VGI and AFLP6/VGII, followed by AFLP5/VGIII, AFLP7/VGIV and AFLP10/VGIV.

AFLP genotyping and mating-type analysis of European* C. gattii *isolates

Seven *C. gattii* isolates were excluded from the European isolates due to their hybrid genetic background. One-hundred European isolates remained and were from clinical (*n*=57; 57%), veterinary (*n*=21; 21%) and environmental (*n*=22; 22%) sources. All environmental isolates belonged to genotype AFLP4/VGI, the ten Italian isolates were mating-type **a** while the 12 isolates from Portugal and Spain were mating-type α. The 21 veterinary cases were obtained from Italy and Spain and belonged to genotype AFLP4/VGI, and four Italian isolates were mating-type **a**, and the remaining 17 isolates, all from Spain, were mating-type α.

The group of 57 clinical isolates was found to be genetically more diverse. All five haploid AFLP genotypes were present, but the largest clusters were formed by genotype AFLP4/VGI (*n*=29) and AFLP6/VGII (*n*=23) isolates. One clinical isolate belonged to genotype AFLP5/VGIII and came from Germany. All clinical *C. gattii* AFLP4/VGI isolates were mating-type α, except for one Italian clinical case that was mating-type **a**. The group of *C. gattii* AFLP6/VGII isolates was divided into the subgenotypes AFLP6A/VGIIa (*n*=6) and AFLP6B/VGIIb (*n*=17), and this genotype cluster contained two mating-type **a** and 21 mating-type α isolates. The two mating-type **a** isolates AV55 and IUM00-5363 came from Greece and were found to be duplicates of the same original isolate. This was also observed for the Greece mating-type α AFLP6B/VGIIb isolate AV54 (the same isolate as IUM01-4731), which was divided into the two phenotypically different isolates 'slimy' and 'white'. Phenotypically different colonies, probably due to phenotypic switching, were also found in other genotypic groups. Within genotype AFLP4/VGI, the original clinical isolates IHEM16633 and IHEM19725 formed 'brown dry' and 'beige slimy' phenotypes. Isolates CBS7952 (AFLP7/VGIV, mating-type α) and IHEM14941 (AFLP10/VGIV, mating-type **a**) were split into CBS7952 'dry' and CBS7952 'slimy' for the former and IHEM14941 'slimy' and IHEM14941 'white' for the latter isolate.

Table 1. Distribution of *C. gattii* isolates by genotype, geographical locality and source.

	AFLP4/ VGI	AFLP5/ VGIII	AFLP6/ VGII	AFLP7/ VGIV	AFLP10/ VGIV	Total	Total (%) (overall)
Africa							
Clinical	18	-	3	8	-	29	
Veterinary	-	-	-	1	-	1	36 (12.0)
Environmental	6	-	-	-	-	6	
Asia							
Clinical	19	-	5	2	-	26	26 (8.6)
Australia							
Clinical	2	1	8	-	-	11	
Veterinary	-	-	1	-	-	1	18 (6.0)
Environmental	5	-	1	-	-	6	
Europe							
Clinical	29	1	23	2	2	57	
Veterinary	21	-	-	-	-	21	100 (33.2)
Environmental	22	-	-	-	-	22	
North America							
Clinical	4	8	18	-	-	30	
Veterinary	2	-	20	-	-	22	73 (24.3)
Environmental	4	5	11	-	-	20	
Unknown	-	-	1	-	-	1	
South America							
Clinical	12	3	10	-	-	25	
Veterinary	1	-	2	-	-	3	33 (11.0)
Environmental	-	-	5	-	-	5	
Unknown							
Clinical	-	2	-	-	-	2	5 (1.7)
Unknown	1	2	-	-	-	3	
Source of isolation							
Clinical	84	15	67	12	2	180 (59.8)	
Veterinary	24	5	23	1	-	53 (17.6)	
Environmental	37	-	17	-	-	54 (17.9)	
Unknown	1	2	1	-	-	4 (1.3)	
All *C. gattii* isolates	146	22	108	13	2	291 (100)	

Table 2. Sequence Type diversity for each of the investigated loci and AFLP genotype clusters.

	CAP10		*CAP59*		*GPD1*		IGS1		*LAC1*		*MPD1*		*PLB1*		*SOD1*		*TEF1α*		*URA5*	
Genotype	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}
AFLP4/VGI (*n*=146)	6	0.499	14	0.609	10	0.504	10	0.504	10	0.540	15	0.667	4	0.514	25	0.776	17	0.506	9	0.627
AFLP5/VGIII (*n*=22)	1	0.567	2	0.567	4	0.805	4	0.805	8	0.848	4	0.732	6	0.749	4	0.403	4	0.823	7	0.779
AFLP6/VGII (*n*=108)	5	0.239	13	0.754	11	0.784	11	0.784	9	0.490	2	0.019	12	0.679	22*	0.809*	3	0.464	11	0.632
AFLP7/VGIV (*n*=13)	4	0.654	9	0.910	5	0.821	5	0.821	3	0.692	1	0.000	4	0.615	7	0.859	5	0.795	2	0.154
All isolates (*n*=291)	19	0.765	40	0.863	32	0.844	52	0.930	31	0.813	25	0.776	30	0.829	59	0.913	34	0.790	30	0.853

	MLST 'Fraser'		MLST 'Meyer'		All MLST loci	
Genotype	n_{ST}	D_{ST}	n_{ST}	D_{ST}	n_{ST}	D_{ST}
AFLP4/VGI (*n*=146)	69	0.908	54	0.926	81	0.945
AFLP5/VGIII (*n*=22)	21	0.996	16	0.974	21	0.996
AFLP6/VGII (*n*=108)	34	0.872	45	0.924	45	0.924
AFLP7/VGIV (*n*=13)	11	0.974	11	0.974	11	0.974
All isolates (*n*=291)	136	0.959	127	0.971	159	0.976

The number of isolates for each of the AFLP genotypic clusters showing the overall distribution, genotype AFLP10/VGIV has been omitted as a separate group from this analysis due to the low number of isolates (*n*=2) that were identical to each other for all ten loci. Sequence Type numbers (n_{ST}) and diversity (D_{ST}) are provided for each locus as well as for the combined loci as previously used by Fraser *et al.* (2005) and Meyer *et al.* (2009) and all loci combined (current study). The study by Fraser *et al.* (2005) included the loci *CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1* and *TEF1α*. The consensus MLST scheme by Meyer *et al.* (2005) included the loci *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5*. The asterisk highlights the absence of four isolates in the analysis of the *SOD1* locus for which no amplicon could be obtained (Supplementary Table 1).

Genetic diversity based on Multi-Locus Sequence Typing

Table 2 listed the sequence type (ST) diversity that was calculated for each of the ten MLST loci and for the combined datasets according to the MLST schemes of Fraser *et al.* (2005) and Meyer *et al.* (2009). When all loci and isolates were included, the *CAP10* locus showed the lowest ST diversity (n_{ST}=19; D_{ST}=0.765) and IGS1 the highest (n_{ST}=52; D_{ST}=0.930). *SOD1* appeared to be the best resolving locus in terms of ST diversity, since it had the highest diversity for AFLP4/VGI and AFLP5/VGIII isolates. In contrast, *MPD1* showed the lowest diversity for AFLP6/VGII and AFLP7/VGIV isolates. The three MLST datasets separated almost all AFLP7/VGIV isolates, resulting in equal D_{ST} values of 0.974. When the three different MLST schemes were compared, the ten loci MLST dataset revealed the highest diversity (n_{ST}=159; D_{ST}=0.976), followed by the consensus seven loci MLST scheme (D_{ST}=0.971) and the seven loci MLST scheme of Fraser *et al.* (2005) with a D_{ST} of 0.959. However, the latter scheme differentiated more STs than the consensus MLST scheme (n_{ST}=136 versus 127).

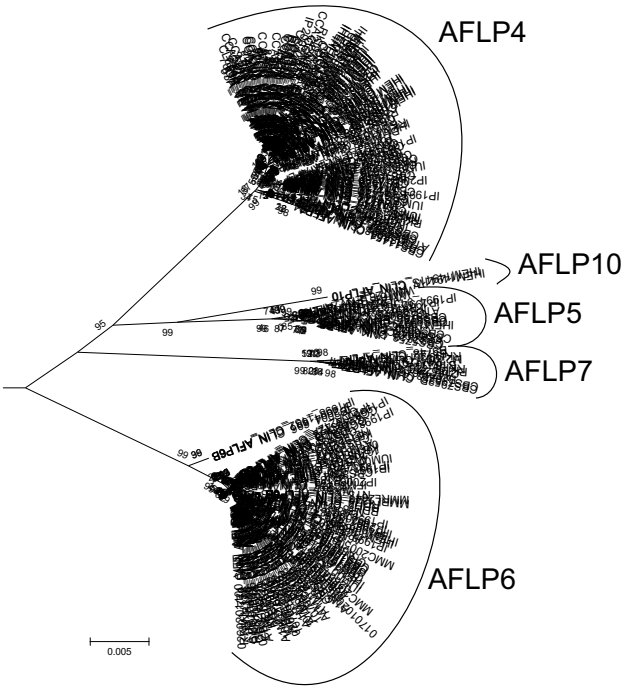

Figure 1. Phylogenetic analysis based on ten loci MLST data of 291 *C. gattii* isolates calculated using the Maximum Likelihood algorithm with 1000 bootstraps and rooted with the two genome sequenced reference *C. neoformans* strains H99 and JEC21 (not shown). The five *C. gattii* AFLP genotypes AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV are highly supported with bootstrap values of ≥95 for the main branches. Subtrees for each of the genotypic clusters are presented in Figure 2 (genotype AFLP4/VGI isolates), Figure 3 (genotype AFLP5/VGIII, AFLP7/VGIV and AFLP10/VGIV isolates) and Figure 4 (genotype AFLP6/VGII isolates).

Phylogenetic analysis and geographic linkage using MLST data

Bootstrap Maximum Likelihood analysis clustered all *C. gattii* isolates together in five highly supported monophyletic clusters, thus supporting their AFLP genotypes (Figure 1). When each cluster was separately analyzed, the branches had low support values of ≤75 (Figure 2-4). The group of genotype AFLP4/VGI isolates had low bootstrap support values for nearly all branches, while the second largest group formed by AFLP6/VGII isolates showed more well supported branches (Figure 2 and 4, respectively).

When the complete ten loci MLST data of the European *C. gattii* isolates was compared with the data obtained from the globally collected *C. gattii* isolates it was observed that genotypic relationships exists between clinical European isolates and those from other geographic origins. The most striking example were the set of four clinical isolates from Danish, Dutch, German and Swiss citizens who developed cryptococcosis after visiting Vancouver Island (Canada). Isolates obtained from these tourists, CBS10485, RKI06/496, RKI01/774 and CBS10866, respectively, had similar AFLP fingerprint pattern and identical MLST profiles as veterinary, clinical and environmental *C. gattii* AFLP6A/VGIIa isolates from the Vancouver Island outbreak (Figure 4). A similar finding was observed for a set of five clinical French *C. gattii* AFLP6B/VGIIb isolates (IP1996/1120-1 and -2, IP1999/901-1 and -2 and IP2000/87) that were genetically indistinguishable from isolate IP2001/935-1 that was obtained from a patient in Senegal (Figure 4). These five French isolates were obtained from patients who had lived before in Africa and immigrated to France. Based on AFLP genotyping three subgenotypes can be discerned (AFLP6A/VGIIa, AFLP6B/VGIIb and AFLP6C/VGIIc), however, the MLST analysis showed that AFLP6A/VGIIa and AFLP6B/VGIIb isolates occurs intermingled in the phylogenetic tree (data not shown), but that the two outbreak related sets of AFLP6A/VGIIa and AFLP6C/VGIIc isolates form both an outbreak specific cluster (Figure 4).

A large proportion of the *C. gattii* AFLP4/VGI isolates from Italy and Spain were found to be genetically homogeneous, irrespective their source of isolation or mating-type background. The Italian clinical isolate *C. gattii* AFLP4/VGI mating-type **a**, which was the reason to perform an environmental and veterinary survey in the living neighborhood of the patient, was found to be identical for all ten MLST loci when compared to the obtained Italian veterinary and environmental *C. gattii* AFLP4/VGI isolates (Figure 2). Strikingly, *C. gattii* AFLP4/VGI isolates involved in numerous small outbreaks among goats in Spain, approximately two decades ago, were genetically indistinguishable from recently obtained clinical, veterinary and environmental isolates from different Spanish provinces as well as from AFLP4/VGI mating-type **a** isolates from Italy (Figure 2). Based on phylogenetic analysis it was observed that these Italian and Spanish isolates belong to a novel Mediterranean *C. gattii* AFLP4/VGI cluster. Some other clinical and environmental AFLP4/VGI isolates were found to have a nearly identical MLST profile (isolates CCA313-316, CCA320, CCA321, CCA326, CCA328, CCA242L, IP2005/215 and RKI1997/482) when compared to the major AFLP4/VGI lineage from Spain and Italy (Figure 2).

A set of isolates from Portugal and Belgium (IP1997/18, IHEM19725B and -S, mating-type α, respectively) was found to be indistinguishable from African (i.e., Congo

AFLP4/VGI
AFLP10/VGIV
AFLP5/VGIII
AFLP7/VGIV
AFLP6/VGII
0.005
AFLP4/VGI subtree
European Mediterranean AFLP4/VGI cluster
53A VET
57A VET
78CN ENV
60A VET
CL6148 CLIN
54A VET
64CN VET
CCA242T CLIN
5UM CLIN
CCA311 CLIN
58A VET
81CN ENV
52A VET
48A VET
30CN ENV
CCA309 VET
CCA308 VET
CCA307 VET
72CN ENV
33CN ENV
66CN ENV
87CN ENV
CCA312 CLIN
51CN VET
84CN VET
50A VET
49A VET
56A VET
CCA232 CLIN
51A VET
42CN VET
75CN ENV
CCA298 VET
CCA314 ENV
CCA326 ENV
CCA315 ENV
CCA328 ENV
CCA316 ENV
CCA242L CLIN
26CN ENV
IP2005/215 CLIN
55A VET
CCA317 ENV
CCA319 ENV
CCA319 ENV
CCA242NEW CLIN
90C ENV
RKI97/482 CLIN
59A VET
CCA313 ENV
CBS6290 CLIN
CCA321 ENV
CCA320 ENV
IHEM11792 CLIN
IHEM16633S CLIN
IHEM16633B CLIN
N114 CLIN
CBS10608 CLIN
CBS10609 CLIN
IUM98-1969 CLIN
IHEM15003 CLIN
RV66095 CLIN
IHEM14915 CLIN
Brazilian cluster
IHEM14956 CLIN
IHEM14955S CLIN
IUM92-6692 CLIN
IHEM14965 CLIN
IHEM14968 CLIN
IHEM14934 CLIN
IHEM14976 CLIN
IHEM14984 CLIN
IHEM11799 CLIN
B16416 CLIN
CBS10600 CLIN
IP2006/958 CLIN
IHEM11795 CLIN
IHEM11798 CLIN
CAB862 ENV
CAB860 ENV
CAB879 ENV
CAB827 ENV
IHEM10602S CLIN
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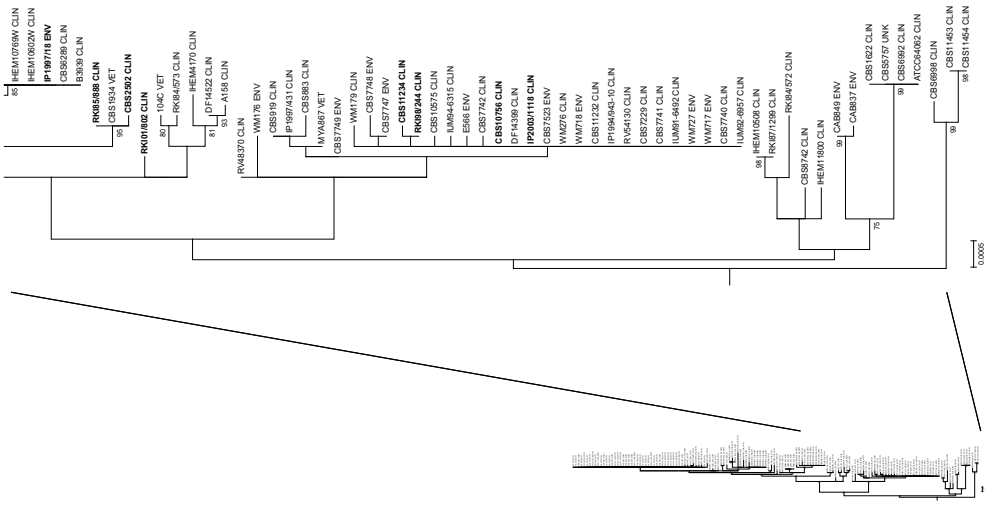
IHEM10769W CLIN



Previous page and above, Figure 2. Phylogenetic tree of *C. gattii* AFLP4/VGI isolates based on ten nuclear MLST-loci as presented in Figure 1 (shown in the left upper corner at the previous page), the bootstrapped Maximum Likelihood phylogenetic analysis has been split into smaller sections as indicated in the overview at the left of each magnified section. Isolates that are within the shaded areas belong to the European Mediterranean or Brazilian *C. gattii* AFLP4/VGI MLST-cluster, as indicated. Isolates from Europe are indicated in bold, the source of the isolates is indicated behind the name of the isolate with 'CLIN' referring to a clinical origin, 'VET' to a veterinary source, 'ENV' to an environmental origin and 'UNK' to an unknown origin. Information of the isolates is listed in Supplementary Table 1.

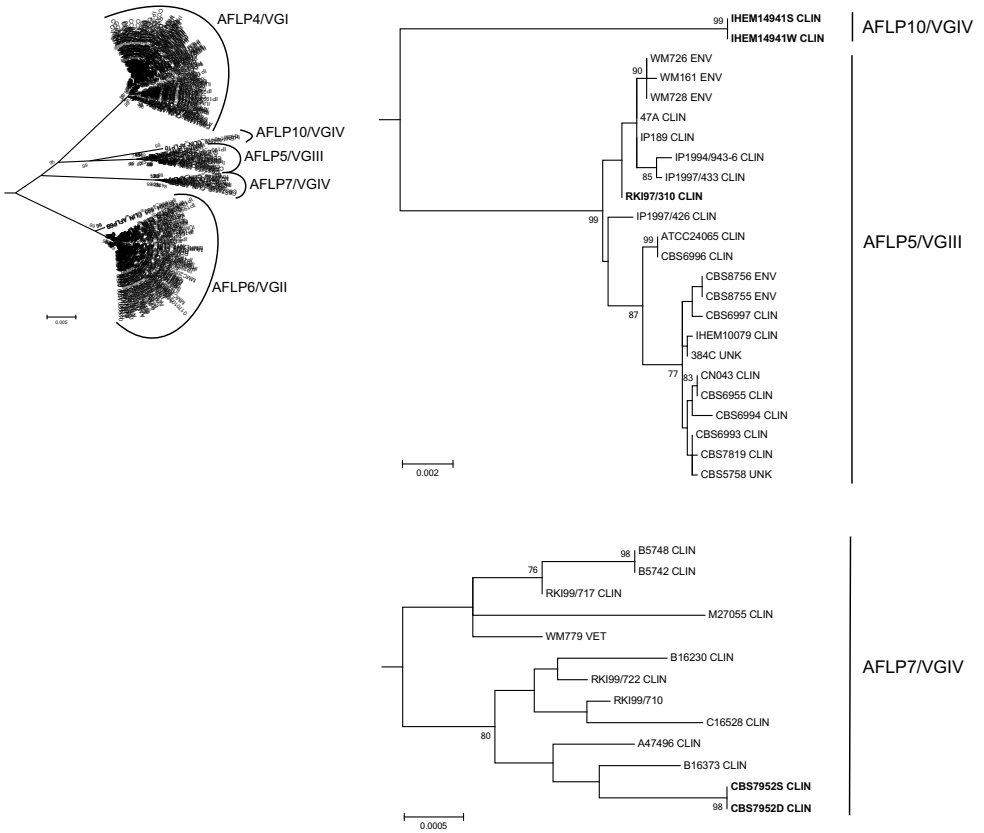
and Rwanda) isolates with a clinical source (B3939 and CBS6289, both mating-type **a**, and IHEM10602S, IHEM10769S and IHEM10769W with mating-type α) (Figure 2). Both sub-isolates of IHEM19725 were obtained from a HIV-infected Rwandese patient who migrated to Belgium.

Various small groups of *C. gattii* AFLP4/VGI isolates with similar MLST profiles were observed, but from different geographic origins. An example are the two clinical isolates CBS2502 and RKI85/888 from the Netherlands and Germany, respectively, that were by MLST analysis indistinguishable from a North American isolate obtained from a cow in 1952 (isolate CBS1934). A second example is the Italian clinical isolate IUM92-6692 obtained from a cryptococcal infection in an immunocompetent Brazilian immigrant, which had an identical MLST profile as six Brazilian clinical mating-type α isolates (IHEM14934, IHEM14956, IHEM14965, IHEM14968, IHEM14976 and IHEM14984) (Figure 2).

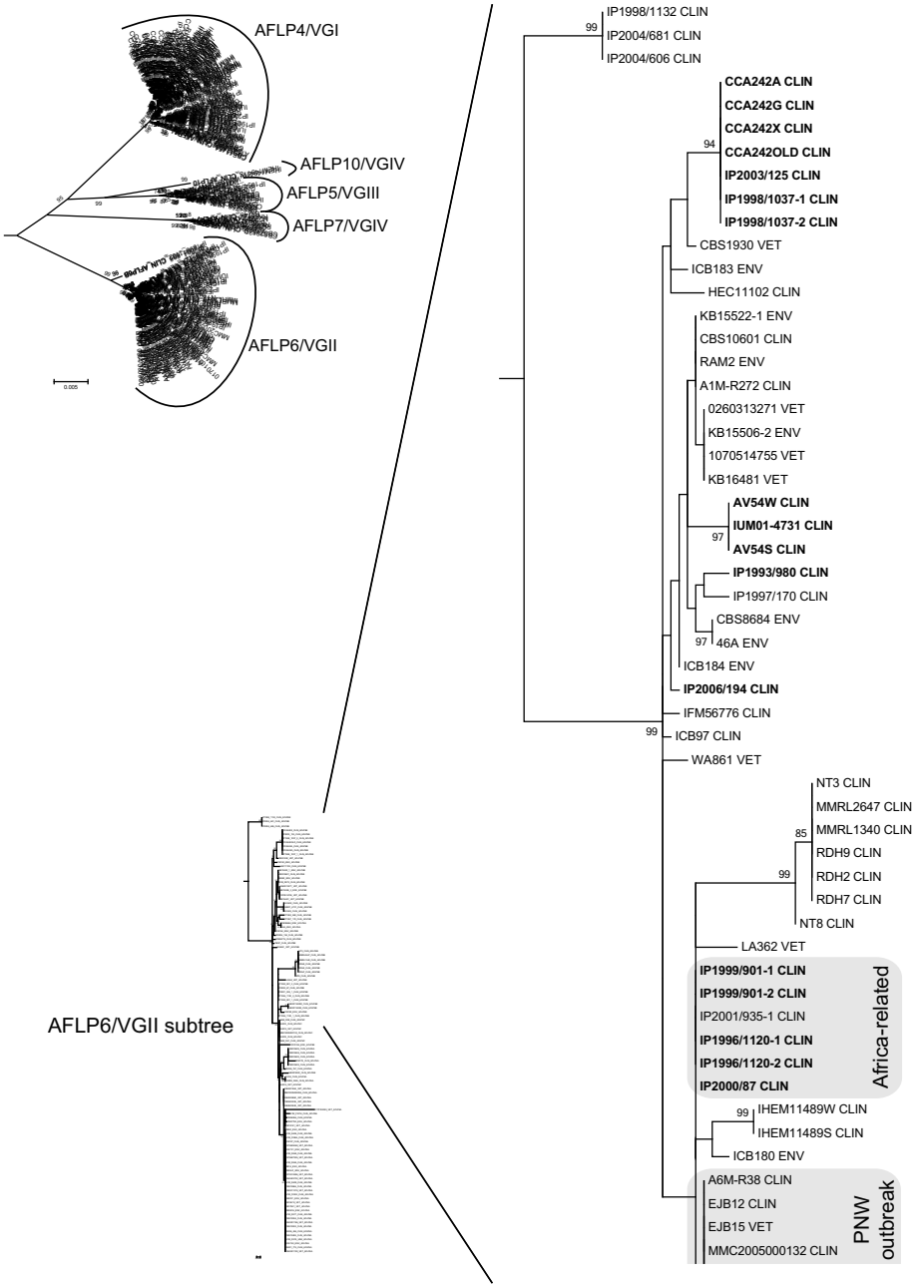
The single *C. gattii* AFLP5/VGIII isolate from a 24-year-old immunocompent German patient (isolate RKI97/310) had a different MLST profile compared to other isolates with the same AFLP fingerprint pattern, but this isolate clustered together with clinical and environmental isolates from Mexico (Figure 3). Interestingly, the case

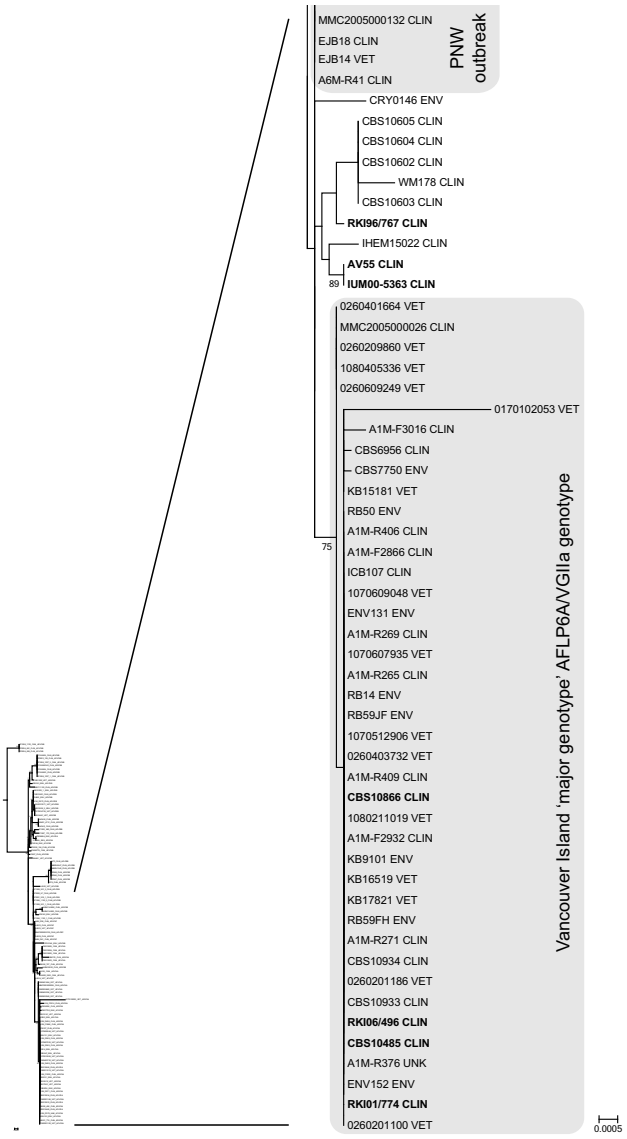
report from this patient revealed that she had travelled to California where *C. gattii* AFLP5/VGIII is endemic (Grosse *et al.*, 2001).

The two *C. gattii* AFLP7/VGIV isolates CBS7952D and CBS7952S, obtained from a Swedish HIV-infected patient, were genetically indistinguishable from each other and had a unique MLST profile that clustered together with Malawian clinical isolates. Years before the onset of her cryptococcal infection, this Swedish patient migrated from Zambia to Sweden (Figure 3).



Above, Figure 3. Maximum Likelihood phylogenetic analysis based on ten loci MLST data of *C. gattii* AFLP5/VGIII, AFLP7/VGIV and AFLP10/VGIV isolates. Subtree of *C. gattii* AFLP5/VGIII, AFLP7/VGIV and AFLP10/VGIV isolates as presented in Figure 1 (shown in the left upper corner). Bootstrapped Maximum Likelihood phylogenetic analyses are shown for the group of AFLP5/VGIII and AFLP10/VGIV isolates (upper panel) and AFLP7/VGIV isolates (lower panel). European isolates are indicated in bold, the source of the isolates is indicated behind the name of the isolate with 'CLIN' referring to a clinical origin, 'VET' to a veterinary source, 'ENV' to an environmental origin and 'UNK' to an unknown origin. Information of the isolates is provided in Supplementary Table 1.


AFLP4/VGI
AFLP10/VGIV
AFLP5/VGIII
AFLP7/VGIV
AFLP6/VGII
0.005
AFLP6/VGII subtree
IP1998/1132 CLIN
IP2004/681 CLIN
IP2004/606 CLIN
CCA242A CLIN
CCA242G CLIN
CCA242X CLIN
CCA242OLD CLIN
IP2003/125 CLIN
IP1998/1037-1 CLIN
IP1998/1037-2 CLIN
CBS1930 VET
ICB183 ENV
HEC11102 CLIN
KB15522-1 ENV
CBS10601 CLIN
RAM2 ENV
A1M-R272 CLIN
0260313271 VET
KB15506-2 ENV
1070514755 VET
KB16481 VET
AV54W CLIN
IUM01-4731 CLIN
AV54S CLIN
IP1993/980 CLIN
IP1997/170 CLIN
CBS8684 ENV
46A ENV
ICB184 ENV
IP2006/194 CLIN
IFM56776 CLIN
ICB97 CLIN
WA861 VET
NT3 CLIN
MMRL2647 CLIN
MMRL1340 CLIN
RDH9 CLIN
RDH2 CLIN
RDH7 CLIN
NT8 CLIN
LA362 VET
IP1999/901-1 CLIN
IP1999/901-2 CLIN
IP2001/935-1 CLIN
IP1996/1120-1 CLIN
IP1996/1120-2 CLIN
IP2000/87 CLIN
Africa-related
IHEM11489W CLIN
IHEM11489S CLIN
ICB180 ENV
A6M-R38 CLIN
EJB12 CLIN
EJB15 VET
MMC2005000132 CLIN
PNW outbreak



Previous page and above, Figure 4. Subtree of *C. gattii* AFLP6/VGII isolates as presented in Figure 1 (shown in the left upper corner at the previous page), the bootstrapped Maximum Likelihood phylogenetic analysis has been split into smaller sections as indicated in the overview at the left of each magnified section. Isolates within shaded areas fell in the indicated clusters of African or the outbreak isolates with genotype AFLP6A/VGIIa (Vancouver Island outbreak cluster) or genotype AFLP6C/VGIIc (Pacific Northwest outbreak cluster). Isolates from Europe are indicated in bold, the source of the isolates is indicated behind the name of the isolate with 'CLIN' referring to a clinical origin, 'VET' to a veterinary source, 'ENV' to an environmental origin and 'UNK' to an unknown origin. Information of the isolates is provided in Supplementary Table 1.

Recombination analysis
Recombination was observed when the clone corrected dataset was used that included all five *C. gattii* AFLP genotypes (P=0.001). Recombination was observed in the clone corrected datasets of genotype AFLP4/VGI (P=0.000), AFLP6/VGII (P=0.000) and AFLP7/VGIV (P=0.015) isolates, while this was not significantly present among the genotype AFLP5/VGIII isolates (P=0.242). No recombination analysis was performed for genotype AFLP10/VGIV since there were only two isolates available.

Discussion

Until a decade ago, *Cryptococcus gattii* was rarely reported as a cause of cryptococcosis outside the endemic localities in tropical and subtropical climate zones in Central Africa, Northern Australia, and South America (Springer & Chaturvedi, 2010). This has been changed due to the emergence of the *C. gattii* outbreak at Vancouver Island (British Columbia, Canada) and its expansion to the mainland of Canada and the further spread into the Pacific Northwest (Byrnes *et al.*, 2010; Datta *et al.*, 2009). This indicates that *C. gattii* might be, or has become more widespread than was thought before.

In Europe *C. gattii* has been found as a cause of apparently autochthonous cryptococcal infections, which has been documented in several case reports. The oldest documented case of a European *C. gattii* infection was made by Curtis (1896), who described an infection in an otherwise healthy young man from Lille, France. Until the 1980s, cryptococcosis was rarely observed and *C. gattii* infections were even more exceptional outside their endemic tropical and subtropical regions. This is highlighted by a case report from 1985 that described a *C. gattii* infection in an immunocompetent German citizen who never travelled abroad Germany (Kohl *et al.*, 1985).

During the past decade, increasing numbers of *C. gattii* infections have been reported in Europe, but so far it remained unclear if these cases were autochthonous or related to travelling to endemic regions (Bodasing *et al.*, 2004; Grosse *et al.*, 2001; Hagen *et al.*, 2007; Velegraki *et al.*, 2001; Viviani *et al.*, 2006). Subclinical dormant *C. gattii* infections have been described and using molecular epidemiological approaches it has been demonstrated that *C. gattii* can be activated upon immune suppression (Dromer *et al.*, 1992; Hagen *et al.*, 2010a). Similarly, it was suggested that a deceased cheetah from the Havana Zoo (Havana, Cuba) was infected by a *C. gattii* isolate that was genetically closely related to African isolates. It was hypothesized that the cheetah developed cryptococcosis, after it was imported in Cuba, due to stress-induced immune suppression while held in captivity (Illnait-Zaragozí *et al.*, 2011).

The current study used a large global collection of *C. gattii* isolates from clinical, veterinary and environmental sources in order to link European isolates with those from other localities. Our study revealed that several European clinical isolates could be linked to endemic regions in Brazil, California and the African continent, thus demonstrating that *C. gattii* can cause infections that remain dormant for years. A fascinating observation is that a large proportion of clinical isolates from Northwestern Europe were acquired from other endemic regions in Africa, North and South America,

while clinical and veterinary isolates from Mediterranean Europe could nearly all be linked to locally collected environmental isolates.

The majority of isolates were obtained from Mediterranean Europe, especially from Spain were during the past two decades several small outbreaks among goats have been observed (Baró *et al.*, 1998; Colom *et al.*, 2011). Nearly all the isolates from the European Mediterranean were found to belong to genotype AFLP4/VGI and MLST analysis showed that within this genotype the Mediterranean isolates form a separate cluster (Figure 2). Interestingly, isolates from Italy with mating-type **a** and those from Spain with mating-type α fell into this Mediterranean MLST-cluster. Although the MLST data indicates a strict clonal propagation of *C. gattii* AFLP4/VGI in the Mediterranean region, the presence of two mating-types suggests that sexual recombination may occur. A similar observation was made in a recent study that involved *C. gattii* AFLP4/VGI isolates from Papua New Guinea that were found to be a clonal population but with mixed mating-types (Campbell *et al.*, 2005). It was hypothesized that inbreeding between genetically closely related isolates occurs but that the apparently clonal structure of this population is not disturbed (Campbell *et al.*, 2005). This might also be the case within the European Mediterranean *C. gattii* AFLP4/VGI population, but it remains remarkable that all mating-type **a** isolates were found in Italy while the Spanish isolates from this cluster were all mating-type α.

Recombination has been detected in the genotypic clusters of globally collected sets of AFLP4/VGI, AFLP6/VGII and AFLP7/VGIV isolates, this supports a recent study that showed recombination events within all *C. gattii* genotypes (Carter *et al.*, 2010; Ngamskulrungroj *et al.*, 2009). However, the observed recombination in the latter and the current study is biased since globally collected isolates for each genotypic cluster were included and it is likely that this introduces false positive signals of recombination (Carvajal-Rodríguez *et al.*, 2006; Saul *et al.*, 2008). A complicating factor that conceal signals of recombination is caused by sexual reproduction between same mating-type isolates, a phenomenon that has been observed in *C. neoformans* and that might be the driving force behind the hypervirulent *C. gattii* AFLP6/VGII outbreak in Canada (Fraser *et al.*, 2005; Wang and Lin, 2011). Recombination has been extensively studied for *C. gattii*, especially for the Australian population. Here it was first observed that an environmental population of *C. gattii* AFLP4/VGI isolates was clonal, based on low genetic diversity and the presence of mating-type α isolates only (Halliday & Carter, 2003). However, follow-up studies observed recombination in AFLP6/VGII and mainly clonal reproduction in AFLP4/VGI Australian isolates (Campbell *et al.*, 2005; Carriconde *et al.*, 2011; Saul *et al.*, 2008). The apparent clonal structure of European Mediterranean *C. gattii* AFLP4/VGI isolates with the presence of both mating-types suggests that recombination might occur here but that it remains invisible, as suggested in the Australian studies (Campbell *et al.*, 2005; Saul *et al.*, 2008).

The fact that *C. gattii* AFLP4/VGI has only been found in the European Mediterranean environment suggests that geographical boundaries exists, either caused by the climate that prevails in this region and/or the Pyrenean and Alps mountains that separates the Iberian Peninsula and Italy from the European mainland. This occurrence in a geographically limited region is further strengthened by the observation that a

proportion of clinical case from Northwestern Europe could be linked to other endemic localities such as Brazil, California, and the African continent, while clinical isolates from Mediterranean Europe are genetically closely related to environmental and veterinary isolates obtained from that region.

In conclusion, MLST analysis based on all ten loci and the seven loci datasets according to Fraser *et al.* (2005) and Meyer *et al.* (2009) revealed that *C. gattii* can be divided into five genotypic clusters, thus supporting previous observations based on other molecular biological typing techniques, such as M13 PCR fingerprinting and AFLP fingerprint analysis (Boekhout *et al.*, 2001; Hagen *et al.*, 2010a; Meyer *et al.*, 2009). MLST has been applied to trace the geographic origin of European clinical and veterinary isolates compared to available environmental isolates. From this we conclude that clinical *C. gattii* infections from Northwestern Europe are mainly acquired outside the European continent, e.g. during visiting an endemic region such as the Vancouver Island outbreak area, or before migration to Europe from endemic regions in Africa and South America. Reactivation of dormant *C. gattii* infections upon immune suppression seems to occur more often than was assumed before, and this indicates that *C. gattii* is more likely to be an opportunistic rather than a primary pathogen, as has been recently suggested (Hagen *et al.*, 2010b; MacDougall *et al.*, 2011).

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Supplementary data

Upon request, Supplementary data will be provided by the authors.

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Chapter 6

Ceratonia siliqua (carob) trees as natural habitat and source of infection by *Cryptococcus gattii* in the Mediterranean environment

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Summary

Recent *Cryptococcus gattii* infections in humans and animals, including several outbreaks in goats, were the basis for an environmental survey in six provinces of Spain. A total of 479 samples from twenty tree species were studied. *Cryptococcus gattii* was found for the first time in autochthonous Mediterranean trees in Spain. Fourteen isolates of this pathogen were obtained from three types of trees: twelve (85.8%) from carob (*Ceratonia siliqua*), one (7.1%) from Mediterranean stone pine (*Pinus halepensis*) and another from eucalyptus (*Eucalyptus camaldulensis*). All *C. gattii* isolates were genotype AFLP4/VGI and mating-type α, and were found to be genetically identical with *C. gattii* strains isolated from humans and animals in Spain. This supports the hypothesis that these trees are the natural source for infection of humans and mammals in the Mediterranean area.

Introduction

Cryptococcus gattii is a basidiomycetous yeast that causes respiratory and disseminated infections in immunocompromised and immunocompetent humans and animals (Sorrell *et al.*, 2001). Hosts are supposed to acquire cryptococci via inhalation of desiccated yeast cells or basidiospores from the environment, and the disease affects the lungs with the potential to disseminate to the central nervous system and/or to other distant tissues (Lin & Heitman, 2006). *Cryptococcus gattii* was traditionally considered as a saprophyte of *Eucalyptus* spp. trees and autochthonous of tropical and subtropical areas (Chakrabarti *et al.*, 1997; Ellis *et al.*, 1990). Since 1999, an outbreak of a hypervirulent *C. gattii* lineage was observed at Vancouver Island in British Columbia (Canada), which expanded recently to the mainland of Canada and the Pacific Northwest (Galanis *et al.*, 2010; Kidd *et al.*, 2004). Since then, previous ideas on epidemiology, infection, ecology and the probable spread of the pathogen from its original environment are under discussion (Datta *et al.*, 2009b; Fraser *et al.*, 2005). Nowadays, *C. gattii* has been isolated from plant debris from a broad range of plant and tree species (Hagen & Boekhout, 2010; Springer & Chaturvedi, 2010).

In Spain, the first cases of cryptococcosis by *C. gattii* occurred as five small outbreaks among goats in the province of Cáceres (Baró *et al.*, 1998). Afterwards, other cases in humans and animals have been reported (Colom *et al.*, 2005; Solla *et al.*, 2008) and several studies were carried out to find *C. gattii* in their environments. Most of these studies focused on eucalyptus trees and were unsuccessful (Torres-Rodríguez *et al.*, 2006).

In 2008, our research group studied a case of cryptococcosis in a ferret and a subsequent environmental study was initiated in Gavà (Barcelona), where the animal lived. Ten samples from carob trees (*Ceratonia siliqua*) and one from a stone pine tree (*Pinus halepensis*) yielded eleven strains of *C. gattii* in this area. After this finding, other carob trees in Alicante (>500km distance) were sampled and two more strains were recovered from four trees (Table 1).

Carob trees belong to the *Fabaceae* family and are perennial leguminous trees native to the Mediterranean basin. Spain harbours the highest number of carob trees in the world but the plant can be found in all Mediterranean countries and in the south of Portugal (Tous *et al.*, 1996). Mediterranean stone pine is also an autochthonous plant of the Mediterranean environment.

This work describes for the first time the presence of *C. gattii* in an autochthonous Mediterranean environmental niche, which is likely to be the source for outbreaks among various animals and humans.

Materials and Methods

Sampling areas

Twenty plant species were sampled in six provinces of Spain: Asturias, Guadalajara, Albacete, Cáceres, Barcelona and Alicante (Figure 1). These provinces are separated by hundreds of kilometres and have different climates and vegetations. Sampled trees included *Eucalyptus camaldulensis* (*n*=158; 33%) that is present in all localities studied and common Mediterranean trees, namely the carob tree (*Ceratonia siliqua*) and the Mediterranean stone pine (*Pinus halepensis*), which were included when sampling the eastern coast of Spain (*n*=123; 25.7% and *n*=39; 8.1%, respectively); holm trees (*Quercus ilex*) (*n*=40; 8.3%) from Cáceres and the Central part of the country (Guadalajara and Albacete); apple trees (*Malus domestica*) from north western Spain, and other minority tree species from different sites. Locations and tree species studied are indicated in Table 1.

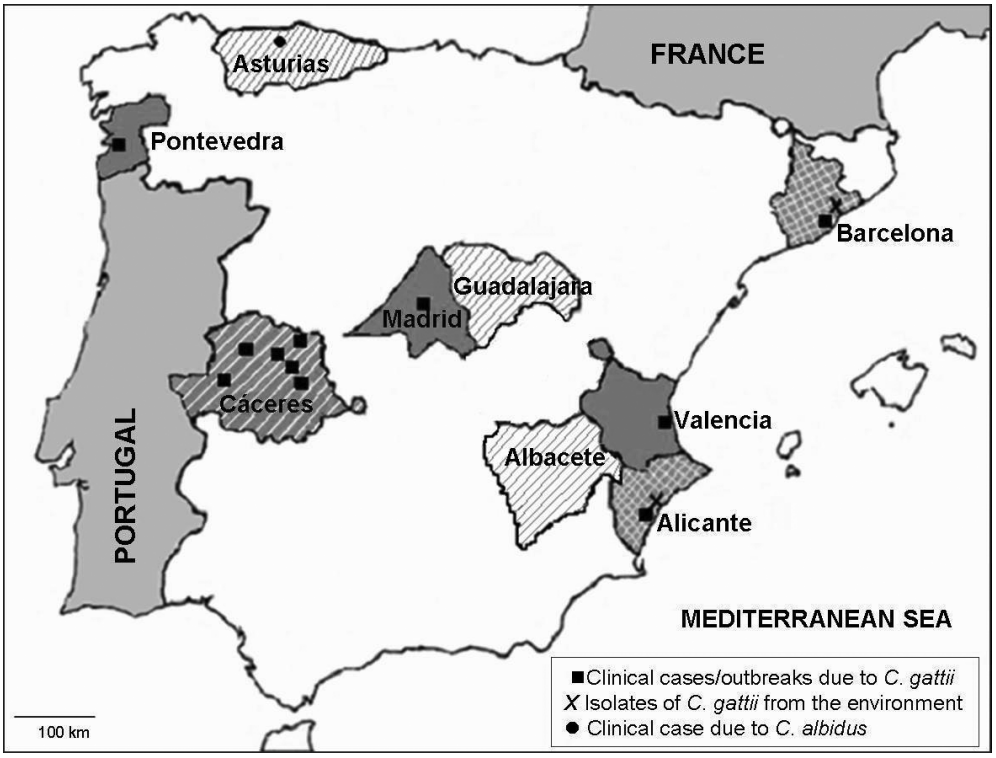


Figure 1. Distribution of *C. gattii* in Spain. Filled areas: provinces where human or veterinarian cases were detected. Striped areas: provinces where environmental samples were collected. Squared areas: both clinical and environmental *C. gattii* were detected.

Table 1. *Cryptococcus* spp. isolated from trees and *C. gattii* strains from clinical specimens.

Locality (trees/samples)	*Cryptococcus* strains	Type of sample	CBS number	Genotype
Carob tree (*Ceratonia siliqua*)				
Barcelona (18/108)	*C. gattii* CCA313	Swab	CBS11813	AFLP4/VGI
	C. gattii CCA314	Swab	CBS11814	AFLP4/VGI
	C. gattii CCA315	Swab	CBS11815	AFLP4/VGI
	C. gattii CCA316	Detritus	CBS11816	AFLP4/VGI
	C. gattii CCA317	Detritus	CBS11817	AFLP4/VGI
	C. gattii CCA319	Swab	CBS11819	AFLP4/VGI
	C. gattii CCA326	Detritus	CBS11826	AFLP4/VGI
	C. gattii CCA328	Detritus	CBS11828	AFLP4/VGI
	C. gattii CCA330	Detritus	CBS11749	AFLP4/VGI
	C. gattii CCA331	Detritus	CBS11750	AFLP4/VGI
	C. neoformans CCA318	Detritus	CBS11818	-
	C. neoformans CCA327	Swab	CBS11827	-
	C. neoformans CCA329	Detritus	CBS11829	-
Alicante (7/15)	*C. neoformans* CCA322	Swab	CBS11822	-
	C. neoformans CCA323	Swab	CBS11823	-
	C. neoformans CCA324	Swab	CBS11824	-
	C. neoformans CCA325	Swab	CBS11825	-
	C. gattii CCA320	Swabs	CBS11820	AFLP4/VGI
	C. gattii CCA321	Bark swab	CBS11821	AFLP4/VGI
Eucalyptus tree (*Eucalyptus camaldulensis*)				
Alicante (55/128)	*C. laurenttii*	Flower	-	-
	C. laurenttii	Fruit	-	-
	C. albidus 10 (no CCA)	-	-	-
	C. albidus CCA255	Swab	-	-
	C. albidus CCA256	Swab	-	-
	C. albidus CCA257	Swab	-	-
	C. albidus CCA258	Swab	-	-
	C. albidus CCA259	Bark	-	-
	C. neoformans CCA249	Detritus	CBS11805	-
	C. albidus CCA250	Bark	-	-
	C. albidus CCA252	Bark	-	-
	C. gattii CCA248	Bark	CBS11748	AFLP4/VGI

Table 1. *continued*

Asturias (10/30)		Bark/leaves/ swabs/soil		
Stone pine tree (*Pinus halepensis*)				
Alicante (10/20)	-	Swabs	-	-
Barcelona (5/19)	*C. gattii* CCA332	Detritus	CBS11751	AFLP4/VGI
Almond tree (*Prunus dulcis*)				
Alicante (14/27)	*C. albidus* CCA253	Bark	-	-
	C. albidus CCA251	Swab	-	-
	C. albidus CCA336	Swab	-	-
	C. albidus CCA337	Swab	-	-
	C. albidus CCA338	Swab	-	-
	C. albidus CCA339	Flower (swab)	-	-
	C. albidus CCA340	Bark	-	-
Judas tree (*Cercis siliquastrum*)				
Alicante (10/20)	*C. albidus* CCA341	Swab	-	-
	C. albidus CCA342	Flower (swab)	-	-
	C. albidus CCA343	Flower (swab)	-	-
Fig tree (*Ficus elastica*)				
Alicante (3/7)	*C. albidus* CCA254	Root	-	-
Olive tree (*Olea europeae*)				
Alicante (1/1)	*C. albidus* CCA263	Swab	-	-
Cáceres (5/5)	–	Swab	-	-
Holm tree (*Quercus ilex*)				
Guadalajara (3/4)	*C. albidus* CCA333	Swab	-	-
Albacete (3/3)	–	Swab	-	-
Cáceres (10/33)	–	Swabs/detritus/ soils/leaves	-	-
Cherry tree (*Prunus* sp.)				
Asturias (3/3)	*C. neoformans* CCA262	Swab	CBS11806	-
Apple tree (*Malus domestica*)				
Asturias (4/4)	*C. neoformans* CCA261	Swab	-	-
Other tree species[1]				
Six provinces (30/52)	–	Swab	-	-

Table 1. *continued*

Veterinary cases (goats)				
Cáceres	*C. gattii* 48A	Lung	CBS11230	AFLP4/VGI
	C. gattii 49A	Lung	CBS11788	AFLP4/VGI
	C. gattii 50A	Lung	CBS11789	AFLP4/VGI
	C. gattii 51A	Lung	CBS11790	AFLP4/VGI
	C. gattii 52A	Liver	CBS11792	AFLP4/VGI
	C. gattii 53A	CSF	CBS11793	AFLP4/VGI
	C. gattii 54A	Lung	CBS11794	AFLP4/VGI
	C. gattii 55A	Lung	CBS11795	AFLP4/VGI
	C. gattii 56A	Brain	CBS11231	AFLP4/VGI
	C. gattii 57A	Lung	CBS11796	AFLP4/VGI
	C. gattii 58A	Lung	CBS11798	AFLP4/VGI
	C. gattii 59A	Lung	CBS11799	AFLP4/VGI
	C. gattii 60A	Lung	CBS11800	AFLP4/VGI
	C. gattii CCA344	Lung	CBS11830	AFLP4/VGI
Veterinary case (ferret)				
Barcelona	*C. gattii* CCA298	Lymph node	CBS11807	AFLP4/VGI
Asymptomatic carriers (ferrets)				
Barcelona	*C. gattii* CCA307	Nasal swab	CBS11808	AFLP4/VGI
	C. gattii CCA308	Nasal swab	CBS11809	AFLP4/VGI
	C. gattii CCA309	Nasal swab	CBS11810	AFLP4/VGI
Clinical cases				
Madrid	*C. gattii* CBS11234	CSF	CBS11234	AFLP4/VGI
Alicante	*C. gattii* CCA232	Brain abcess	CBS11728	AFLP4/VGI
Pontevedra	*C. gattii* CBS11752	CSF	CBS11752	AFLP4/VGI
Asymptomatic carriers (humans)				
Barcelona	*C. gattii* CCA311	Nasal swab	CBS11811	AFLP4/VGI
	C. gattii CCA312	Nasal swab	CBS11812	AFLP4/VGI

Collection and processing of samples

A total of 479 samples were obtained from twenty tree species. Samples consisted of swabs from trunk and hollows (*n*=339; 70.7%); bark pieces (*n*=24; 5.0%), leaves (*n*=16; 3.3%), flowers (*n*=35; 7.3%), fruits (*n*=5; 1.1%), detritus (*n*=44; 9.2%), soil under the tree canopy (*n*=11; 2.3%) and others (*n*=5; 1.1%). Approximately 200 grams of soil samples and plant debris were collected into sterile plastic containers. For most bark samples,

hollows and other tree surfaces, the swabbing technique proposed by Randhawa and co-workers (2005) was used. For plant debris, approximately 5 grams of samples were processed as described before (Granados *et al.*, 2005; Randhawa *et al.*, 2005).

Identification of Cryptococcus *isolates*

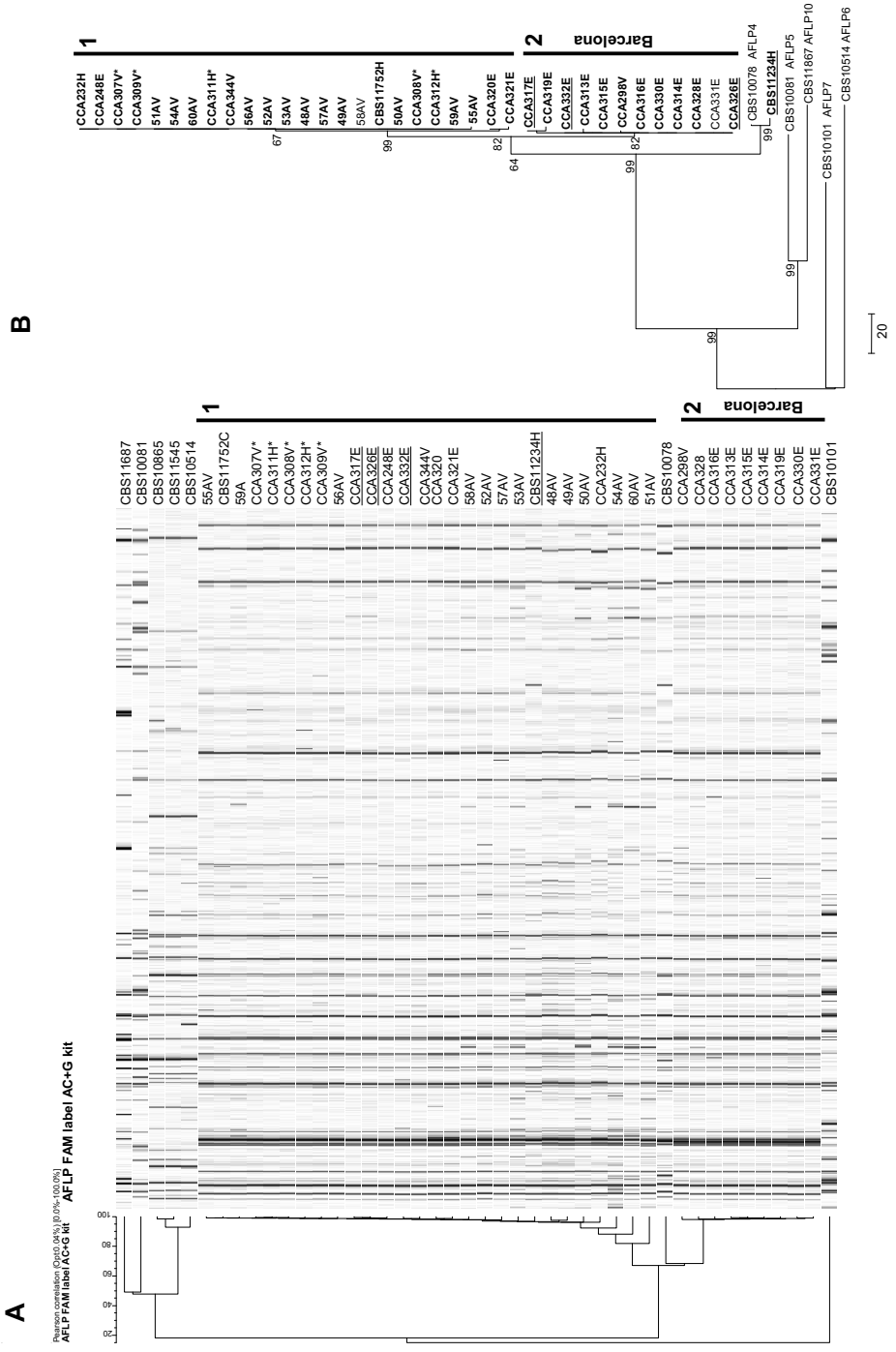
All yeast isolates were subjected to physiological and morphological identification tests in order to identify the *Cryptococcus* species. Performed tests included cell and colony morphology, ability to grow at 37°C, hydrolysis of urea, presence of capsule by Indian ink negative staining, colorimetric sugar assimilation (Auxacolor Bio-Rad, Marnes-la-Coquette, France), actidione sensitivity, phenoloxidase production and ability to grow on L-canavanine-glycine-bromothymol blue medium (Kwon-Chung *et al.*, 1982). The isolated *Cryptococcus* spp. strains were stored in our collection (CCA, Coleccion *Cryptococcus* Alicante) and all *C. gattii* and *C. neoformans* isolates were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre (Table 1).

Genetic analysis of C. gattii *isolates*

Cryptococcus gattii isolates were further studied using Amplified Fragment Length Polymorphism (AFLP) and Multi-Locus Sequence Typing (MLST) to compare them with strains from previous cases of cryptococcosis in humans and animals in Spain (Table 1).

Genomic DNA was extracted as described previously (Hagen *et al.*, 2010). AFLP fingerprint analysis was carried out as described previously (Boekhout *et al.*, 2001). AFLP genotypes were assigned using the following reference isolates that were included in the analysis: CBS10078 (AFLP4/VGI); CBS10081 (AFLP5/VGIII); CBS10514 (AFLP6A/VGIIa); CBS10865 (AFLP6B/VGIIb); CBS11545 (AFLP6C/VGIIc); CBS10101 (AFLP7/VGIV) and CBS11687 (AFLP10/VGIV) (Hagen *et al.*, 2010). Subsequently, ten nuclear loci were amplified and sequenced according to recently published methods (Meyer *et al.*, 2009). Seven of these loci are part of a recently launched MLST scheme that includes the *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1* and *URA5*, while the *CAP10*, *MPD1* and *TEF1α* were sequenced to compare the strains with a previously published large *C. gattii* MLST study (Fraser *et al.*, 2005; Meyer *et al.*, 2009). Raw sequence data was analyzed using SeqMan v8.0.2 (DNASTAR, Madison, WI) followed by an automatic alignment and bootstrap Maximum Parsimony phylogenetic analysis using MEGA v4.1 (Kumar *et al.*, 2008). The Maximum Parsimony tree was obtained using the Close-Neighbor-Interchange algorithm with a search level 3 in which the initial trees were obtained with the random addition of sequences (ten replicates). The tree was drawn to scale with branch lengths calculated using the average pathway method and are in the

Next page, Figure 2. Epidemiological relatedness of Spanish environmental and clinical *C. gattii* strains. Panel A shows the genetic relatedness based on AFLP fingerprint analysis, Panel B shows the phylogenetic analysis based on MLST data of ten unlinked nuclear loci, using the Maximum Parsimony algorithm. The source of the *C. gattii* strains is indicated as environmental (E), veterinary (V) and human (H). Asterisks indicate isolates from asymptomatic carriers. Underlined *C. gattii* isolate numbers are showing differences in MLST and AFLP clustering. Bootstrap values are indicated next to the branches.


A
Pearson correlation (Opt:0.04%) [0.0%-100.0%]
AFLP FAM label AC+G kit
AFLP FAM label AC+G kit
1
2
Barcelona
CBS11687
CBS10081
CBS10865
CBS11545
CBS10514
CBS11752C
CCA307V*
CCA311H*
CCA308V*
CCA312H*
CCA309V*
CCA317E
CCA326E
CCA248E
CCA332E
CCA344V
CCA320
CCA321E
CBS11234H
CBS10078
CCA298V
CCA328
CCA316E
CCA313E
CCA315E
CCA314E
CCA319E
CCA330E
CCA331E
CBS10101
B
CCA232H
CCA248E
CCA307V*
CCA309V*
CCA311H*
CCA344V
CBS11752H
CCA308V*
CCA312H*
CCA320E
CCA321E
CCA317E
CCA319E
CCA332E
CCA313E
CCA315E
CCA298V
CCA316E
CCA330E
CCA314E
CCA328E
CCA331E
CCA326E
CBS10078 AFLP4
CBS11234H
CBS10081 AFLP5
CBS11867 AFLP10
CBS10101 AFLP7
CBS10514 AFLP6
20

units of the number of changes over the whole concatenated set of ten sequenced loci.

The mating-type of each isolate was determined using *C. gattii* specific primers for either the mating-type **a** or α *STE12* locus, reference isolates CBS10078 (mating-type α) and CBS1930 (mating-type **a**) were included as controls (Hagen *et al.*, 2010).

Results

Cryptococcus gattii was found in two of the six sampled provinces of Spain, namely Barcelona and Alicante, both situated at the Mediterranean coast and separated by more than 500 kilometres (Figure 1). Clinical cases due to *C. gattii* were reported in both provinces (Colom *et al.*, 2005; N. Morera, unpublished observations). In Alicante *C. gattii* was detected on two different tree species: one strain (CCA248) was obtained in 2005 from a sample of *Eucalyptus camaldulensis* bark while two others (CCA320 and CCA321) were isolated during the summer of 2009 from swabs of the trunk of carob trees. In Barcelona, all isolates were obtained in the park of Gavà, where a *C. gattii* veterinary case was reported a few months before (N. Morera, unpublished observations). In this park, *C. gattii* was found in ten samples from carob trees and one sample of detritus from a stone pine tree. All these samples were collected during the summer of 2009.

The study yielded 56 *Cryptococcus spp.* isolates identified as *C. albidus* (*n*=31; 55.4%), *C. gattii* (*n*=14; 25.0%), *C. neoformans* (*n*=9; 16.1%) and *C. laurentii* (*n*=2; 3.6%). The majority of *C. gattii* and *C. neoformans* strains were isolated from carob trees (*n*=12; 85.7% and *n*=7; 77.8%, respectively). Only two other trees harboured *C. gattii*: eucalyptus and Mediterranean stone pine (*n*=1; 7.2% each).

Cryptococcus gattii was found in twelve out of the 123 carob tree related samples (9.8%). Six *C. gattii* strains were collected from detritus of carob and stone pine trees and another six strains were obtained from swabs from the tree surface and hollows in the trunks of carob trees. Nine strains of *C. neoformans* were isolated from three different tree species (Table 1). Carob trees harboured the highest number of detected *C. neoformans* with seven (77.8%) strains (Table 1). The other two strains were found in eucalyptus and a cherry tree (both 11.1%). *Cryptococcus albidus* was the most abundant *Cryptococcus* species among the studied samples. It appeared in seven out of the twenty sampled tree species (35%): eucalyptus, fig tree, almond tree, judas tree, olive tree, holm tree and apple tree yielded in total 31 isolates of this species. The two *C. laurentii* isolates from *Eucalyptus* sp. were obtained during a previous study in Alicante city (Colom-Valiente *et al.*, 1997). All the isolates, type of sample and sampled trees species are listed in Table 1.

Environmental strains that were identified as *C. gattii* using CGB medium were subjected to Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis and Multi-Locus Sequence Typing (MLST) to study their genetic relatedness with previously described clinical and veterinary isolates (Table 1; Figure 2). This revealed that Spanish *C. gattii* strains fell in the same cluster as reference strain CBS10078, representing genotype AFLP4/VGI isolates. AFLP analysis divided the Spanish *C. gattii* strains in two clusters: a major cluster that contained a group of fourteen veterinary

isolates, three isolates from human cases of cryptococcosis and six environmental strains from different trees. Also five *C. gattii* isolates fell into this cluster that were isolated from asymptomatic carriers (three ferrets and two humans) from Barcelona. The minor AFLP cluster contained a veterinary strain from Barcelona which was 97.9% identical to eight environmental *C. gattii* strains isolated from carob trees (*Ceratonia siliqua*) (Figure 2).

Phylogenetic MLST analysis, based on ten unlinked nuclear loci (6083nt aligned with 39 parsimony informative sites, including 31 singletons), revealed a nearly identical topology. However three strains (CCA317, CCA326 and CCA332) were now clustering together with the minor AFLP cluster, which was due to four shared polymorphisms in the *SOD1* locus (Figure 2). The previously published *C. gattii* MLST dataset from (Fraser *et al.*, 2005) containing the *CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1* and *TEF1α* loci, was used to compare the Spanish *C. gattii* isolates. This showed that Spanish *C. gattii* AFLP4/VGI isolates had a distinct MLST profile compared to those found previously (Fraser *et al.*, 2005; Supplementary Figure 1). Sequences were submitted to Genbank and are available under accession number HM622758-HM623127.

Discussion

The present study describes for the first time the presence of the emerging pathogen *Cryptococcus gattii* in the Mediterranean environment. The isolation of the yeast from twelve samples of carob trees (*Ceratonia siliqua*) located in two geographically separated provinces of Spain could be considered as evidence for a Mediterranean niche for *C. gattii.*

Eucalyptus trees only yielded one strain of each pathogenic *Cryptococcus* species. This low rate of isolation is in accordance with previous findings from the Mediterranean area that were initiated in Egypt, Italy, Jordan, Spain and Turkey after reports of clinical *C. gattii* infections (Baró *et al.*, 1998; Campisi *et al.*, 2003; Colom-Valiente *et al.*, 1997; Ergin *et al.*, 2004; Hagen & Boekhout, 2010; Hamasha *et al.*, 2004; Mahmoud *et al.*, 1999; Montagna *et al.*, 1997; Torres-Rodríguez *et al.*, 2006). These studies focused mainly on samples from eucalyptus trees. Only in Egypt and Italy the species was detected in 1.2% and 2.4% of the samples, respectively (Mahmoud *et al.*, 1999; Montagna *et al.*, 1997).

The present study reports a high rate of positive *C. gattii* samples that were retrieved from carob trees (9.8%). Similar rates were reported from Colombia and India where the overall presence of *C. gattii* in trees was estimated to range from 2.6% to 16.1%, respectively (Granados *et al.*, 2006; Randhawa *et al.*, 2011).

All environmental, human and veterinary *C. gattii* strains from Spain were serotype B, mating-type α and genotype AFLP4/VGI. Two lineages were observed when a phylogenetic comparison was made based on AFLP fingerprint patterns and MLST results using all ten nuclear loci (Figure 2). When the Spanish *C. gattii* were compared to those used in a previous global MLST study (Fraser *et al.*, 2005) it was observed that there was no differentiation between the two Spanish *C. gattii*-clusters (Supplementary

Figure 1). This shows that for epidemiological studies a genome-wide fingerprinting method might be a valuable additional tool to provide more detailed information compared to techniques such as MLST and PCR fingerprint typing that shows only differences in aforehand selected nuclear loci. The difference between the seven and ten nuclear based MLST analysis shows that the addition of a particular locus, in this case *SOD1*, contributes to subtle differences in the phylogenetic analysis as shown by the split of the Spanish *C. gattii* isolates in two clusters, nearly similar to those found by AFLP fingerprint analysis (Figure 2).

The majority of Spanish clinical, veterinary and environmental isolates were genotypically identical, thus suggesting clonal dispersal. The identical genotypes of the investigated strains strongly suggest that animals and humans, who suffered from cryptococcosis in the Mediterranean area, may have acquired *C. gattii* from environmental sources such as trees in their neighbourhood. Interestingly enough, the genotype of the isolates from humans and goats located in the West and Northwest of the country, were found to be also identical but, the carob tree is not known to grow in these areas. Thus the ecological source for these *C. gattii* infections still remains to be determined.

The list of worldwide reported tree species that harbours *C. gattii* includes over fifty species (Kidd *et al.*, 2007; Springer & Chaturvedi, 2010). From these findings, major evidence accumulated and showed the existence of a close relationship between trees and yeasts of the *Cryptococococcus* genus. Whether *C. gattii* is more frequently found in trees than *C. neoformans*, and why some tree species are more suitable to harbor these yeasts should be further addressed. The production of laccase and some abiotic factors, such as pH, humidity and temperature, seem to be important for the distribution and ecology of *Cryptococcus* species (Botes *et al.*, 2009; Datta *et al.*, 2009a; Granados *et al.*, 2006; Grover *et al.*, 2007; Zhu & Williamson, 2004). Biochemical compounds in bark, flowers and other debris of plants, may play an important role in the specificity of the association of a *Cryptococcus* species to a specific tree or group of trees. The isolation of high numbers of *C. albidus* isolates in the current study is in agreement with another recent study from Malaysia (Tay *et al.*, 2008).

From the current results, it can be concluded that carob trees are an important environmental niche for *C. gattii* in the Mediterranean area. The presence of the yeast in these trees may explain the occurrence of clinical and veterinary *C. gattii* cases reported before. More Mediterranean zones and trees should be sampled and biological and biochemical environmental aspects should be taken into consideration to assess the existence of a stable yeast-tree relationship.

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Supplementary data

Supplementary data is only available at http://informahealthcare.com/doi/suppl/10.3109/13693786.2011.574239.

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Chapter 7

In vitro antifungal susceptibilities and Amplified Fragment Length Polymorphism genotyping of a worldwide collection of 350 clinical, veterinary and environmental *Cryptococcus gattii* isolates

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Summary

The *in vitro* susceptibility of a worldwide collection of 350 *Cryptococcus gattii* strains was tested for seven antifungal drugs including the new triazole isavuconazole. With Amplified Fragment Length Polymorphism (AFLP) fingerprinting, human, veterinary and environmental *C. gattii* strains were subdivided into seven AFLP genotypes including the interspecies hybrids AFLP8 and AFLP9. The majority of 215 clinical strains comprised genotypes AFLP4 (n=76) and AFLP6 (n=103). The clinical AFLP6 strains had a significant higher geometric mean minimal inhibitory concentration (MIC) for 5-flucytosine and fluconazole compared to the clinical AFLP4 strains. Of the seven antifungal compounds examined in this study, isavuconazole had the lowest MIC_{90} (0.125 µg/ml) among all *C. gattii* strains, followed by a one log_2 dilution step (MIC_{90} 0.25 µg/ml) by itraconazole, voriconazole and posaconazole. Amphotericin B had an acceptable MIC_{90} of 0.5 µg/ml, but fluconazole and 5-flucytosine had relatively high MIC_{90}s of 8 µg/ml.

Introduction

The basidiomycetous yeast *Cryptococcus gattii* is responsible for life threatening invasive disease in apparently healthy humans and animals (Bovers *et al.*, 2008a; Ma & May, 2009). A typical *C. gattii* infection is acquired through the respiratory tract from where it can further disseminate to the central nervous system resulting in fatal meningitis (Bovers *et al.*, 2008a; Ma & May, 2009; Velagapudi *et al.*, 2009). Cryptococcosis caused by the primary pathogenic yeast *C. gattii* was, until a decade ago, a rarely encountered infection outside tropical and subtropical regions (Kwon-Chung & Bennett, 1984; Sorrell, 2001; Springer & Chaturvedi, 2010). However, this changed due to an unprecedented outbreak that emerged in the temperate climate of Vancouver Island (British Columbia, Canada) that subsequently expanded further into the Pacific Northwest (Bartlett *et al.*, 2008; Byrnes *et al.*, 2009; Datta *et al.*, 2009; Kidd *et al.*, 2004. Its sibling species *C. neoformans* differs ecologically and epidemiologically from *C. gattii* since it occurs on a global scale and is linked with disease occurring in immunocompromised individuals, such as HIV-positive patients and transplant patients who receive immune-suppressive medicines (Bovers *et al.*, 2008a; Datta *et al.*, 2009; Kwon-Chung *et al.*, 1982; Ma & May, 2009; Van Elden *et al.*, 2000).

Cryptococcus gattii can be discerned from *C. neoformans* using a wide range of microbiological and molecular techniques (Bovers *et al.*, 2008a; Meyer *et al.*, 2009). A convenient method is the use of L-canavanine-glycine-bromothymol blue (CGB) medium, which allows *C. gattii*, in contrast to *C. neoformans*, to grow and changing the pH indicator in the medium from green-yellowish to blue (Kwon-Chung *et al.*, 1982). With the increasing use of molecular techniques, such as PCR fingerprinting, Restriction Fragment Length Polymorphism analysis of the *PLB1* and *URA5* locus, Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis, as well as with several Multi-Locus Sequence Typing (MLST) approaches, it became clear that *C. gattii* could be divided into five distinct genotypes named AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV and the recently observed novel genotype AFLP10 (Boekhout *et al.*, 2001; Bovers *et al.*, 2008a, 2008c; Hagen *et al.*, 2009; Kidd *et al.*, 2004; Meyer *et al.*, 2003, 2009; Ngamskulrungroj *et al.*, 2009). Until recently, a serotype agglutination assay was widely used to distinguish *C. neoformans* (serotype A and D) from *C. gattii* (serotype B and C) (Bovers *et al.*, 2008a; Springer & Chaturvedi, 2010). In general, serotype B strains are found in each of the five *C. gattii* AFLP genotypes, but it seems that *C. gattii* serotype C strains are restricted to the genotypes AFLP5/VGIII and AFLP7/VGIV (Boekhout *et al.*, 2001; Bovers *et al.*, 2008c; Kidd *et al.*, 2004; Meyer *et al.*, 2003; Springer & Chaturvedi, 2010).

In addition it was found that *C. gattii* and *C. neoformans* can form interspecies hybrids, named genotype AFLP8 (*C. neoformans* var. *neoformans* AFLP2/VNIII serotype D × *C. gattii* AFLP4/VGI serotype B) and AFLP9 (*C. neoformans* var. *grubii* AFLP1/VNI serotype A × *C. gattii* AFLP4/VGI serotype B). These interspecies hybrids are, until now, only isolated from clinical samples, and they might have a higher virulence potential than regular *C. gattii* or *C. neoformans* isolates (Bovers *et al.*,

2006, 2008b; F. Hagen, K. Tintelnot and T. Boekhout, unpublished data).

Treatment of cryptococcosis depends, besides the immune status of the patient, on the severity and localization of the infection (Dromer *et al.*, 2008). Severe cases of cryptococcosis in immunocompetent and -compromised patients are treated according to the guidelines of the Infectious Diseases Society of America (IDSA) which consists of an induction therapy for two weeks with a combination of amphotericin B and 5-flucytosine, followed with a ten weeks consolidation therapy with fluconazole (Dromer *et al.*, 2008; Perfect *et al.*, 2010).

Cryptococcus neoformans has been extensively studied for its *in vitro* susceptibility for a wide variety of antifungal compounds, including the new triazoles posaconazole, voriconazole, ravuconazole and isavuconazole (Gomez-Lopez *et al.*, 2008; Illnait-Zaragozí *et al.*, 2008; Thompson *et al.*, 2008, 2009; Yamazumi *et al.*, 2000). Despite the ongoing *C. gattii* outbreak, only a few studies using relatively small sets of *C. gattii* isolates, have been performed to investigate the *in vitro* susceptibility for amphotericin B, 5-flucytosine, fluconazole and the new triazole antifungals (Gomez-Lopez *et al.*, 2008; Iqbal *et al.*, 2010; Thompson *et al.*, 2008, 2009; Trilles *et al.*, 2004). A few studies divided the *C. gattii* isolates into groups according to their serotype or genotype (Iqbal *et al.*, 2010; Thompson *et al.*, 2009).

Therefore, we studied the *in vitro* susceptibility of each of the *C. gattii* genotypes from a large worldwide collection, subdivided by AFLP genotyping, for amphotericin B, 5-flucytosine, fluconazole, itraconazole, voriconazole, posaconazole, and the new experimental broad-spectrum antifungal triazole isavuconazole.

Materials and methods

Strains and media

A worldwide collection of 350 *C. gattii* strains, representing 215 clinical (61.4%), 57 veterinary (16.3%), 73 environmental (20.9%) and 5 strains from an unknown source (1.4%), were included in this study. Strains were obtained from the public culture collections of the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands), BCCM-IHEM Scientific Institute of Public Health (Brussels, Belgium) as well as the working group collections of the authors. According to their geographical origin, *C. gattii* strains were grouped as Africa (n=28; 8.0%), Asia (n=22; 6.3%), Australia (n=20; 5.7%), Europe (n=101; 28.9%), North America (n=95; 27.1%), South America (n=81; 23.1%) and unknown origin (n=3; 0.9%). The group of North American strains contained a set of 75 (24.1%) *C. gattii* strains related to the ongoing Vancouver Island outbreak. Distribution of the *C. gattii* strains, according to their geographical origin and source of isolation, is listed in Table 1. Strains were checked for purity and cultivated on Malt Extract Agar medium (MEA; Oxoid, Basingstoke, United Kingdom). After inoculation, the cultures were incubated for two days at 30°C. A working collection was made by growing *C. gattii* strains on MEA slants for two days at 30°C after which the strains were stored at 4°C.

Table 1. Distribution of *C. gattii* isolates based on AFLP genotype, geographical origin, clinical, veterinary and environmental source.

	Geographical origin							Source of isolation				
AFLP genotype	**African (%)**	**Asian (%)**	**Australia (%)**	**Europe (%)**	**North America (%)**	**South America (%)**	**Unknown origin (%)**	**Clinical source (%)**	**Veterinary source (%)**	**Environmental source (%)**	**Unknown source (%)**	**Total (%)**
AFLP4	16 (11.7)	14 (10.2)	8 (5.8)	74 (54.0)	9 (6.6)	15 (10.9)	1 (0.7)	76 (55.5)	29 (21.2)	30 (21.9)	2 (1.5)	137 (39.1)
AFLP5	-	-	1 (4.5)	2 (9.1)	10 (45.5)	7 (31.8)	2 (9.1)	15 (68.2)	-	5 (22.7)	2 (9.1)	22 (6.3)
AFLP6	3 (1.8)	6 (3.6)	11 (6.5)	19 (11.2)	75 (44.4)	55 (32.5)	-	103 (60.9)	27 (16.0)	38 (22.5)	1 (0.6)	169 (48.3)
AFLP6A*	-	4 (5.5)*	1 (1.4)*	5 (6.8)*	56 (76.7)*	7 (9.6)*	-	36 (49.3)*	18 (24.7)*	18 (24.7)*	1 (1.4)*	73 (43.2)*
AFLP6B*	3 (3.4)*	2 (2.3)*	10 (11.4)*	14 (15.9)*	11 (12.5)*	48 (54.5)*	-	61 (69.3)*	7 (8.0)*	20 (22.7)*	-	88 (52.1)*
AFLP6C*	-	-	-	-	8 (100)*	-	-	6 (75.0)*	2 (25.0)*	-	-	8 (4.7)*
AFLP7	7 (63.6)	2 (18.2)	-	2 (18.2)	-	-	-	10 (90.9)	1 (9.1)	-	-	11 (3.1)
AFLP8	2 (33.3)	-	-	2 (33.3)	-	2 (33.3)	-	6 (100)	-	-	-	6 (1.7)
AFLP9	-	-	-	2 (66.7)	1 (33.3)	-	-	3 (100)	-	-	-	3 (0.9)
AFLP10	-	-	-	-	-	2 (100)	-	2 (100)	-	-	-	2 (0.6)
Total (%)	28 (8.0)	22 (6.3)	20 (5.7)	101 (28.9)	95 (27.1)	81 (23.1)	3 (0.9)	215 (61.4)	73 (20.9)	57 (16.3)	5 (1.4)	350 (100)

An asterisk indicates strains that belong to one of the subgenotypes within AFLP6, namely AFLP6A, AFLP6B or AFLP6C, these strains are cumulative the number of strains in the main genotype AFLP6 group.

Genomic DNA extraction

A previously described extraction procedure by Bolano and colleagues (2001) was further optimized to obtain high quality genomic DNA. *Cryptococcus gattii* strains were subcultured for 2 days at 30°C on Yeast-extract (1%) Peptone (1%) D-Glucose (2%) Agar supplemented with 0.5 M NaCl to prevent the formation of a polysaccharide capsule. Approximately 150 µl of *C. gattii* cells were incubated for 3 hours in 1.6 ml urea solution (10.7 M) at room temperature. After centrifugation at 4000 × *g* for 2 minutes, the pellet was resuspended in 750 µl of lysis buffer (10% w/v sodium dodecyl sulphate, 10% w/v sodium sarkosyl in Tris-EDTA buffer, pH 8.0) and 750 µl phenol:chloroform:isoamyl-alcohol (25:24:1, pH 8.0). Cells were disrupted by bead beating at full speed (TissueLyser, Qiagen, Venlo, The Netherlands) for 5 minutes. After centrifugation at 17000 × *g* for 15 minutes at 4°C, approximately 750 µl supernatant was added to an equal volume of ice cold 96.2% ethanol and 100 µl ice cold 3.0M sodium acetate. Precipitation of genomic DNA was accelerated by incubating the solution at -20°C for 1 hour. Genomic DNA was precipitated by centrifugation at 17000 × *g* for 15 minutes at 4°C and the obtained pellet was dissolved in 100 µl Tris-EDTA buffer (pH 8.0) and 1 µl RNase (Invitrogen, Breda, The Netherlands) was added, followed by an incubation step at 37°C to degrade any remaining RNA. Quality and quantity of genomic DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) and final working solutions with a concentration of 100 ng/µl were prepared.

Mating-, sero- and genotyping

The mating-type of all strains was determined by partly amplification of the *STE12* locus, of either the mating-type **a** or mating-type α allele using two different primer sets, as described by Bovers and co-workers (2006). Briefly, 1 µl genomic DNA (100 ng/µl) was added to 24 µl PCR mixture consisting of 17.8 µl ddH_2O, 0.75 µl $MgCl_2$ (50 mM; Bioline, London, United Kingdom), 2.5 µl 10× PCR Buffer (Bioline), 1.9 µl dNTP's (1 mM; Bioline),0.1 µl Taq DNA polymerase (5 U/µl; Bioline) and 0.5 µl of the forward and reversed primer (Biolegio, Nijmegen, The Netherlands) for either the *STE12*α- (STE12α-F809 5'-TTGACCTTTTRTTCCGCAATG-3' and STE12α-R1607 5'-TTTCTTCTCCCCTGTTTATAGGC-3') or *STE12***a**-allele (STE12a-F537 5'-GTTCTTTGGAATGGCTTATTTCATAT-3' and STE12a-R1299 5'- GMCTTGCGTGGATC ATATCTA-3'). PCR reactions were carried out with an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, followed by 72°C for minutes and finally cooled down to 21°C. As a control, *C. gattii* mating-type α strains CBS6956 and WM276 (= CBS10510) and mating-type **a** strains CBS1930 and E566 (= CBS11233) were included.

The Cryptocheck Iatron agglutination test kit (Iatron Laboratories, Tokyo, Japan) was used to determine the serotype for those *C. gattii* strains with contradictory serotype data.

The genotype of each *C. gattii* isolate was determined using Amplified Fragment Length Polymorphisms (AFLP) fingerprint analysis as described previously by Boekhout and co-workers (2001). The *C. gattii* strains WM178 (= CBS10082; AFLP6/

VGII), A1M-R265 (= CBS10514; AFLP6A/VGIIa), A1M-R272 (= CBS10865; AFLP6B/VGIIb), A6M-R38 (= CBS11545; AFLP6C/VGIIc), WM161 (= CBS10081; AFLP5/VGIII), WM179 (= CBS10078; AFLP4/VGI), WM779 (= CBS10101; AFLP7/VGIV), CBS10488 (AFLP8), CBS10496 (AFLP9) and IHEM14941S (= CBS11687; AFLP10) were used as controls to assign the AFLP genotypes to all *C. gattii* strains (Boekhout *et al.*, 2001; Byrnes *et al.*, 2009; Hagen *et al.*, 2009; Meyer *et al.*, 2003, 2009).

In vitro *susceptibility testing*

The minimal inhibitory concentrations (MIC) of amphotericin B (Bristol Myers Squibb, Woerden, The Netherlands), 5-flucytosine (Valeant Pharmaceuticals, Zoetermeer, The Netherlands), fluconazole and voriconazole (Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (Janssen Cilag, Tilburg, The Netherlands), posaconazole (Schering-Plough, Kenilworth, NJ), and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) were determined using the broth microdilution method, in accordance with the guidelines of the CLSI document M27-A3 (Clinical and Laboratory Standards Institute, 2008). Drug-free and sterile controls were included. *Paecilomyces variotii* (ATCC 22319) and *Candida krusei* (ATCC6258 = CBS573) were used as a quality control. Microtiter plates were incubated for 72h at 35°C under aerobic conditions.

Data analysis

Values for MIC_{50} and MIC_{90} were obtained by ordering MIC data for each antifungal in ascending arrays and selecting the median and 90th quantile, respectively, of the MIC distribution. Geometric mean MICs were computed using Microsoft® Office Excel 2007 (Microsoft, Redmond, WA), for which purpose values "<X" were set equal to "0.5 X" and ">Y" were set equal to "2Y". A two-tailed Mann-Whitney-Wilcoxon test was applied to compare the MIC values between different *C. gattii* genotypes, a multivariate analysis of variance (MANOVA) test was applied to compare geographical origin, AFLP genotype profile and MIC values (StatistiXL v1.8, StatistiXL, Nedland, WA, Australia). A *P*-value of ≤0.05 was regarded as statistically significant.

Results

Mating-, sero- and genotyping

Based on the AFLP genotype, the 350 *C. gattii* strains were divided into five haploid (*n*=341; 97.4%) and two hybrid AFLP genotypes (*n*=9; 2.6%), namely AFLP4 (*n*=137; 39.1%), AFLP5 (*n*=22; 6.3%), AFLP6 (*n*=169; 48.3%), AFLP7 (*n*=11; 3.1%), AFLP8 (*n*=6; 1.7%), AFLP9 (*n*=3; 0.9%) and AFLP10 (*n*=2; 0.6%). The majority of the 215 clinical *C. gattii* strains belonged to either genotype AFLP4 (*n*=76; 35.4%) or AFLP6 (*n*=103; 47.9%). Strains that clustered within genotype AFLP6 could be further divided into the three subgenotypes AFLP6A (*n*=73; 43.2%), AFLP6B (*n*=88; 52.1%) and AFLP6C (*n*=8; 4.7%) (Table 1).

The serotypes of the genotype AFLP7 strains were investigated in more detail since several of these strains were, according to the literature, known to be serotype B

Table 2. MIC range, MIC_{50}, MIC_{90} and geometric mean MIC (µg/ml) for all *C. gattii* AFLP genotypes.

		MIC (µg/ml)			
	Drug	**Range**	**MIC_{50}**	**Geometric Mean**	**MIC_{90}**
All *C. gattii* strains (*n*=350)	Amphotericin B	<0.016 - 1	0.25	0.272	0.5
	5-Flucytosine	0.125 - >64	2	2.896	8
	Fluconazole	0.25 - 64	4	3.573	8
	Itraconazole	<0.016 - 1	0.125	0.124	0.25
	Voriconazole	<0.016 - 1	0.125	0.108	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.113	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.051	0.125
***C. gattii* AFLP4 (*n*=137)**	Amphotericin B	<0.016 - 0.5	0.25	0.238	0.5
	5-Flucytosine	0.125 - 16	1	1.667	4
	Fluconazole	0.25 - 16	2	2.550	8
	Itraconazole	<0.016 - 0.5	0.125	0.119	0.25
	Voriconazole	<0.016 - 0.25	0.063	0.090	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.123	0.25
	Isavuconazole	<0.016 - 0.5	0.031	0.044	0.125
***C. gattii* AFLP5 (*n*=22)**	Amphotericin B	0.125 - 0.5	0.25	0.214	0.5
	5-Flucytosine	0.5 - 4	2	1.938	4
	Fluconazole	0.25 - 8	2	2.064	4
	Itraconazole	<0.016 - 0.25	0.063	0.078	0.125
	Voriconazole	<0.016 - 0.125	0.063	0.059	0.125
	Posaconazole	0.031 - 0.25	0.063	0.076	0.125
	Isavuconazole	<0.016 - 0.063	0.031	0.023	0.063
***C. gattii* AFLP6 (*n*=169)**	Amphotericin B	0.125 - 1	0.25	0.312	0.5
	5-Flucytosine	0.5 - >64	4	5.033	8
	Fluconazole	0.5 - 64	4	5.137	16
	Itraconazole	<0.016 - 1	0.125	0.148	0.5
	Voriconazole	<0.016 - 1	0.125	0.142	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.119	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.067	0.25

Table 2. *continued*

***C. gattii* AFLP6A (*n*=73)**	Amphotericin B	0.125 - 0.5	0.25	0.299	0.5
	5-Flucytosine	1 - 16	4	5.268	8
	Fluconazole	0.5 - 32	4	4.235	8
	Itraconazole	<0.016 - 1	0.125	0.123	0.5
	Voriconazole	<0.016 - 1	0.125	0.117	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.105	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.050	0.25
***C. gattii* AFLP6B (*n*=88)**	Amphotericin B	0.125 - 1	0.25	0.319	0.5
	5-Flucytosine	0.5 - >64	4	4.609	8
	Fluconazole	1 - 16	8	5.438	16
	Itraconazole	<0.016 - 0.5	0.25	0.162	0.25
	Voriconazole	<0.016 - 0.5	0.125	0.152	0.25
	Posaconazole	<0.016 - 0.25	0.125	0.124	0.25
	Isavuconazole	<0.016 - 0.25	0.063	0.078	0.25
***C. gattii* AFLP6C (*n*=8)**	Amphotericin B	0.25 - 0.5	0.25	0.354	0.5
	5-Flucytosine	4 - 16	8	8.724	16
	Fluconazole	8 - 64	16	16.000	64
	Itraconazole	0.25 - 0.5	0.25	0.297	0.5
	Voriconazole	0.25 - 1	0.25	0.354	1
	Posaconazole	0.125 - 0.5	0.25	0.229	0.5
	Isavuconazole	0.063 - 0.5	0.25	0.193	0.5
***C. gattii* AFLP7 (*n*=11)**	Amphotericin B	0.125 - 0.5	0.5	0.343	0.5
	5-Flucytosine	1 - 8	2	2.269	8
	Fluconazole	0.5 - 32	4	4.537	16
	Itraconazole	<0.016 - 0.25	0.125	0.104	0.25
	Voriconazole	0.031 - 0.25	0.125	0.117	0.25
	Posaconazole	0.031 - 0.25	0.125	0.110	0.25
	Isavuconazole	<0.016 - 0.25	0.063	0.052	0.125

Table 2. *continued*

***C. gattii* AFLP8-10**	Amphotericin B	0.125 - 0.5	0.25	0.220	0.25
(*n*=11)	5-Flucytosine	1 - 8	1	1.656	8
	Fluconazole	0.5 - 8	2	2.130	8
	Itraconazole	<0.016 - 0.125	0.063	0.043	0.125
	Voriconazole	<0.016 - 0.25	0.031	0.059	0.125
	Posaconazole	<0.016 - 0.125	0.063	0.043	0.125
	Isavuconazole	<0.016 - 0.063	0.016	0.020	0.063

and C (Bovers *et al.*, 2008a; Meyer *et al.*, 2003; Ngamskulrungroj *et al.*, 2009). However, we determined all *C. gattii* AFLP7 strains as serotype C. The serotype of the two genotype AFLP10 strains was determined to be serotype B. Within the other AFLP genotypes, no contradictions from the literature were found and serotyping was omitted for these groups.

The mating-type was determined for all *C. gattii* strains by amplification of either the *STE12***a** or *STE12*α alleles. This revealed that within the group of 341 haploid *C. gattii* strains (genotype AFLP4-7, 10) the mating-type α strains (*n*=296; 86.8%) were dominantly present compared to the mating-type **a** (*n*=45; 13.2%).

In vitro *susceptibility testing*

MIC ranges, MIC_{50}s, geometric mean MICs, and MIC_{90}s related to AFLP genotype are presented in Table 2. For each antifungal for each AFLP genotype examined, the MIC_{50} and geometric mean MIC values differed by <1 $\log_2$ dilution step, indicating that in all cases the MIC_{50}s obtained by inspection reasonably reflect the central tendency of antifungal susceptibility of the population.

Isavuconazole had the lowest MIC_{90}s across all *C. gattii* surveyed by at least one $\log_2$ dilution step. The overall range of MICs for each of the seven drugs was <0.016 – 1 µg/ml for amphotericin B, 0.125 – >64 µg/ml for 5-flucytosine, 0.25 – >64 µg/ml for fluconazole, <0.016 – 1 µg/ml for itraconazole and voriconazole, and <0.016 – 0.5 µg/ml for posaconazole and isavuconazole. The geometric mean MIC values varied widely when calculated for all 350 *C. gattii* strains (Table 2). The highest geometric mean MICs were 3.573 µg/ml for fluconazole, followed by 2.896 µg/ml for 5-flucytosine, 0.272 µg/ml for amphotericin B, and much lower geometric mean MICs for itraconazole and the new triazoles, voriconazole, posaconazole and isavuconazole (0.124, 0.108, 0.113 and 0.051 µg/ml, respectively).

Comparison of geometric mean MIC values of the *C. gattii* strains obtained from clinical, environmental and veterinary sources revealed that the latter category of strains had a decreased susceptibility for all seven investigated antifungal drugs, except 5-flucytosine, in comparison to clinical and environmental strains. These differences were significant for itraconazole (*P*=0.026; *P*=0.02) and the novel triazoles posaconazole (*P*=0.008; *P*=0.02), voriconazole (*P*=0.013; *P*=0.008) and isavuconazole (*P*=0.004;

Table 3. MIC range, MIC_{50}, MIC_{90} and geometric mean MIC for all *C. gattii* geographic groups.

	Drug	Range	MIC_{50}	Geometric Mean	MIC_{90}
African *C. gattii*	Amphotericin B	0.125 - 0.5	0.25	0.320	0.5
(*n*=28)	5-Flucytosine	1 - 16	2	2.378	8
	Fluconazole	0.25 - 32	4	4.308	8
	Itraconazole	<0.016 - 0.25	0.125	0.111	0.25
	Voriconazole	<0.016 - 0.25	0.125	0.114	0.25
	Posaconazole	<0.016 - 0.25	0.125	0.122	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.058	0.125
Asian *C. gattii*	Amphotericin B	0.125 - 0.5	0.25	0.312	0.5
(*n*=22)	5-Flucytosine	0.5 - 16	2	2	8
	Fluconazole	0.5 - 16	2	2.741	8
	Itraconazole	0.031 - 0.5	0.125	0.1	0.250
	Voriconazole	0.031 - 0.25	0.063	0.08	0.250
	Posaconazole	0.063 - 0.5	0.125	0.107	0.250
	Isavuconazole	0.016 - 0.25	0.031	0.044	0.125
Australian *C. gattii*	Amphotericin B	0.125 - 0.5	0.25	0.297	0.5
(*n*=20)	5-Flucytosine	1 - 32	2	3.138	16
	Fluconazole	0.5 - 16	2	2.639	8
	Itraconazole	0.063 - 0.5	0.125	0.134	0.25
	Voriconazole	0.031 - 0.25	0.125	0.091	0.25
	Posaconazole	0.031 - 0.5	0.125	0.121	0.25
	Isavuconazole	0.016 - 0.125	0.063	0.049	0.125
European *C. gattii*	Amphotericin B	<0.016 - 1	0.25	0.223	0.5
(*n*=101)	5-Flucytosine	0.125 - >64	2	2.263	8
	Fluconazole	0.5 - 32	4	3.301	8
	Itraconazole	<0.016 - 0.5	0.125	0.137	0.25
	Voriconazole	<0.016 - 1	0.125	0.112	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.131	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.055	0.125

Table 3. *continued*

North American *C. gattii*	Amphotericin B	0.125 - 0.5	0.25	0.300	0.5
(*n*=95)	5-Flucytosine	1 - 16	4	4.334	8
	Fluconazole	0.5 - 64	4	4.240	8
	Itraconazole	<0.016 - 1	0.125	0.121	0.25
	Voriconazole	<0.016 - 1	0.125	0.114	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.103	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.047	0.25
South American *C. gattii*	Amphotericin B	0.125 - 1	0.25	0.277	0.5
(*n*=81)	5-Flucytosine	0.5 - 16	4	2.865	8
	Fluconazole	0.25 - 16	4	3.548	16
	Itraconazole	<0.016 -1	0.125	0.126	0.25
	Voriconazole	<0.016 - 0.5	0.125	0.111	0.25
	Posaconazole	<0.016 - 0.5	0.125	0.104	0.25
	Isavuconazole	<0.016 - 0.5	0.063	0.051	0.25

P=0.002) when clinical versus veterinary and environmental versus veterinary *C. gattii* strains were compared, respectively.

A multivariate analysis of variance test (MANOVA) showed that there was a significant difference (*P*<0.001) between susceptibility values obtained from the seven antifungal compounds versus the geographical origin of the *C. gattii* strains (Table 3). It was observed that the MIC values of amphotericin B and 5-flucytosine contributed to this significant difference (*P*=0.003; *P*=0.02, respectively). This difference was caused by *C. gattii* strains from Europe that differed significantly in their susceptibility for amphotericin B compared to strains from Africa (*P*=0.002), Asia (*P*=0.014), Australia (*P*=0.041), North America (*P*<0.001) and South America (*P*=0.019) that were found to be less susceptible. For 5-flucytosine, North American *C. gattii* strains had a significantly higher geometric mean MIC compared to that of African (*P*<0.001), Asian, (*P*<0.001), European (*P*<0.001) and South American (*P*=0.001) strains (Table 3). When all *C. gattii* genotype AFLP4 and AFLP6 strains (Table 2) were analyzed by MANOVA, it was observed that not the geographic origin but the genotype contributed to the significant differences in antifungal susceptibilities patterns (*P*<0.001). The antifungal compounds amphotericin B (*P*<0.001), fluconazole (*P*<0.001), itraconazole (*P*=0.038), voriconazole (*P*=0.001) and isavuconazole (*P*=0.001) were found to be highly significant contributors to this phenotypic difference between AFLP4 and AFLP6 *C. gattii* strains.

Clinical strains that belonged to the genotype AFLP6 cluster had a significant (*P*<0.05) higher geometric mean MIC than strains with genotype AFLP4 for six of the seven tested antifungal compounds. However, both genotypes had an almost identical

geometric mean MIC for posaconazole (0.112 versus 0.124 µg/ml; *P*=0.391). Strikingly, clinical AFLP6 strains had a high geometric mean MIC of 4.961 and 5.638 µg/ml for 5-flucytosine and fluconazole, respectively, compared to clinical strains within genotype AFLP4 (1.401 and 2.467 µg/ml, respectively).

C. gattii strains within the Vancouver Island outbreak-related genotype AFLP6 were divided into the three defined subgenotypes AFLP6A (*n*=73; 43.2%), AFLP6B (*n*=88; 52.1%) and AFLP6C (*n*=8; 4.7%). Significant differences in the geometric mean MICs between *C. gattii* AFLP6A and AFLP6B strains were found for all antifungal drugs, except for amphotericin B (*P*=0.398) and 5-flucytosine (*P*=0.091), however, geometric mean MICs were within one $\log_2$ dilution step. There was also no significant difference in amphotericin B MIC when AFLP6A and AFLP6C (*P*=0.353) or AFLP6B and AFLP6C (*P*=0.607) were compared, while all other tested antifungal compounds showed significant differences between these subgenotypes (Table 2). However, the number of strains within subgenotype AFLP6C is too small to compare the antifungal susceptibility characteristics with the larger group of strains within genotype AFLP6A and AFLP6B.

Discussion

Despite the fact that *C. gattii* has become an important primary pathogen due to the expanding outbreak in British Columbia (Canada) and the Pacific Northwest (USA), there is a lack of in-depth studies on the susceptibility of large sets of *C. gattii* strains against conventional and new antifungal compounds (Datta *et al.*, 2009). Especially the relation between susceptibility profiles and the different genotypes within the *C. gattii* complex has rarely been studied and only with small numbers of isolates (Iqbal *et al.*, 2010). In this study we have compared the susceptibility of 350 globally collected *C. gattii* strains from different sources (e.g. clinical, veterinary and environmental) against four clinically used antifungal compounds i.e. amphotericin B, 5-flucytosine, fluconazole, itraconazole and the three novel triazoles voriconazole, posaconazole and isavuconazole. The older antifungal drugs amphotericin B, 5-flucytosine and fluconazole had the least activity against all *C. gattii* strains, compared to itraconazole and the novel triazoles, as visualized by their geometric mean MIC values (Table 2). The observation that 5-flucytosine and fluconazole had a poorer antifungal activity against *C. gattii*, as well as against its sibling *C. neoformans*, was a consistent finding in previous studies (Gomez-Lopez *et al.*, 2008; Illnait-Zaragozí *et al.*, 2008; Iqbal *et al.*, 2010; Pfaller *et al.*, 2005; Thompson *et al.*, 2009; Trilles *et al.*, 2004).

For susceptibility testing of *C. neoformans* against amphotericin B, 5-flucytosine and fluconazole, so-called 'surrogate breakpoints' were introduced by Pfaller and colleagues (2005). *Cryptococcus neoformans* was regarded highly susceptible for amphotericin B when the MIC was *in vitro* ≤1 µg/ml. In this study none of the 350 *C. gattii* strains showed higher *in vitro* MICs and thus remain all (highly) susceptible. Similar findings were reported in three other studies with lower numbers of tested isolates (Iqbal *et al.*, 2010; Thompson *et al.*, 2009; Trilles *et al.*, 2004). On the contrary,

decreased *in vitro* susceptibility (≥16 µg/ml) for fluconazole was observed for 26 strains (7.4%) and only one clinical isolate within the AFLP6C cluster appeared to be *in vitro* 'high-level resistant' (≥64 µg/ml) for fluconazole. With respect to 5-flucytosine, the number of strains within the group of 'decreased susceptible' (≥8 µg/ml) was 84 (24.3%) of which 9 (2.57%) belong to the category of 'high-level resistant' strains (≥32 µg/ml).

The majority of *C. gattii* strains in the present study belong to either genotype AFLP4 (*n*=137; 39.1%) or AFLP6 (*n*=169; 48.3%), while the remaining 44 (12.6%) *C. gattii* strains belong to one of the minor genotypes (AFLP5, 7-10) that are more restricted to certain geographic regions and cause less frequently infections (Bovers *et al.*, 2008a; Springer & Chaturvedi, 2010). The preponderance towards *C. gattii* strains with an AFLP4 or AFLP6 genotype profile can be interpreted as being a non-representative selection of strains. This apparent over-representation might be due to the inclusion of preserved culture collection strains from well studied geographic localities such as Australia and the *C. gattii* outbreak region in North America (Springer & Chaturvedi, 2010). These localities were repeatedly found to harbor more genotype AFLP4 or AFLP6 strains rather than strains with genotype AFLP5 or AFLP7 (Iqbal *et al.*, 2010; Kidd *et al.*, 2004; Springer & Chaturvedi, 2010). The South American continent has been shown to be a melting-pot of known haploid AFLP genotypes compared to other localities (Meyer *et al.*, 2003; Ngamskulrungroj *et al.*, 2009; Springer & Chaturvedi, 2010). However, preserved *C. gattii* strains from the less well studied regions Africa, Asia and Europe were found to follow the trend that strains with an AFLP4 genotype profile were more frequently isolated than others (Hagen *et al.*, 2009; Springer & Chaturvedi, 2010). Hence, it appears that *C. gattii* AFLP4 and AFLP6 strains are collected and preserved obviously more frequently and that availability and exchangeability of strains can influence studies towards a specific genotype (Hagen *et al.*, 2009; Springer & Chaturvedi, 2010). It might influence the outcome of susceptibility values when only the geographical origin is taken into consideration, while a combined analysis of the geographical origin with a genotyping method (e.g. RFLP, AFLP or MLST) will reveal more subtle differences as has been shown in the current study.

When only the clinical *C. gattii* strains from the two major genotypes AFLP4 and AFLP6 were compared, it appeared that strains belonging to AFLP6 (*n*=103) had a significant ($P<0.05$) higher geometric mean MIC for six of the tested antifungal compounds than strains with genotype AFLP4 (*n*=76). However, both genotypes had an almost identical geometric mean MIC for posaconazole (0.112 versus 0.124 µg/ml). Strikingly, clinical AFLP6 strains had high geometric mean MICs of 4.961 and 5.638 µg/ml for 5-flucytosine and fluconazole, respectively, compared to clinical strains within genotype AFLP4 (1.401 and 2.467, respectively). Similar results were recently reported in a collection of 43 North American *C. gattii* isolates (Iqbal *et al.*, 2010). In the current study, fluconazole and 5-flucytosine had the lowest activity against *C. gattii* strains. Itraconazole, which has been used clinically to treat cryptococcosis, had a MIC_{90} of only 0.25 µg/ml, which is one or two $\log_2$ dilutions lower than found previously by others (Iqbal *et al.*, 2010; Trilles *et al.*, 2004). Isavuconazole, posaconazole, and voriconazole demonstrated excellent potency against each strain and all AFLP genotypes, including strains with reduced fluconazole susceptibilities.

Voriconazole results were comparable (MIC_{90} 0.25 µg/ml) to other studies (Iqbal *et al.*, 2010; Thompson *et al.*, 2009; Trilles *et al.*, 2004) and one log_2 dilution higher in one study (Morera-López *et al.*, 2005). Posaconazole MICs differed a maximum of two log_2 dilution steps. Isavuconazole had the lowest MIC_{90} (0.125 µg/ml) in this large study and was within one log_2 dilution (MIC_{90} 0.063 µg/ml) comparable with results recently reported by Thompson and colleagues (2009). When tested by E-test, these authors also found a MIC_{90} of 0.125 µg/ml (28). Isavuconazole has even a better *in vitro* activity against *C. neoformans*, a finding which holds also true for the other antifungal compounds (Illnait-Zaragozí *et al.*, 2008).

All tested antifungal compounds were active against all *C. gattii* genotypes, however, some strains showed high MICs, as was the case for amphotericin B (up to 1 µg/ml), 5-flucytosine (>64 µg/ml), fluconazole (64 µg/ml), itraconazole and voriconazole (1 µg/ml). The two novel triazoles isavuconazole and posaconazole had, compared to the other tested antifungal compounds, relatively low MIC values. We conclude that amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole were the most active drugs *in vitro* against *C. gattii* strains. Isavuconazole, with its attractive pharmacological and toxicological properties and availability in oral and parenteral formulations, may be a welcome addition to the clinician's antifungal armamentarium to fight this pathogen.

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Chapter 8

Out of the Amazon rainforest – ancient dispersal of the human fungal pathogen *Cryptococcus gattii*

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Summary

Several fungal outbreaks have emerged during the past two decades causing serious and often fatal diseases in humans and other animals. Among the most prominent of these outbreaks is the expanding Vancouver Island *Cryptococcus gattii* outbreak that has affected hundreds of otherwise healthy humans and animals, including several fatal cases. During the past decade, *C. gattii* has caused outbreaks on several other localities outside the tropical and subtropical where it was observed before. Remarkably, these outbreaks were found to be all caused by a previously rarely found genotype of *C. gattii* called AFLP6/VGII, while none of the other four *C. gattii* genotypes has been observed to be actively involved in an outbreak.

Several attempts were made to find the geographic origin of the *C. gattii* AFLP6 lineage in order to trace the genetic origin of the hypervirulent trait of the strains involved in the Vancouver Island outbreak, however, this had led to different and opposite conclusions. To solve the dilemma, we have undertaken extensive molecular analysis of the *C. gattii* AFLP6/VGII lineage using a combination of Amplified Fragment Length Polymorphisms (AFLP) fingerprinting, a novel multi-locus microsatellite panel and highly discriminative coalescence gene genealogy analysis of Multi-Locus Sequence Typing scheme based on Sequence Characterized AFLP Regions data. In addition, we have carried out additional sequencing and re-analysis of a recently published MLST.

These analyses revealed that South American *C. gattii* AFLP6/VGII strains represent the ancestral lineages within the global population, in agreement with the observed high genetic diversity at that locality. Coalescence gene genealogy analysis revealed that the basal lineages within genotype AFLP6/VGII have a South American origin, providing strong evidence that South America is the cradle for the global population as well as for the expanding *C. gattii* AFLP6/VGII Vancouver Island and the Pacific Northwest outbreaks. Furthermore, we show that the South American *C. gattii* population has undergone frequent historical recombination. Thus the hypervirulent *C. gattii* AFLP6/VGII outbreak lineage has likely been derived from a regular mating event in South America that gave rise to hypervirulent offspring that was able to settle in more temperate climate zones where it is able to colonize and disperse at higher rates via a yet unknown genetic mechanism.

Introduction

During recent decades, a number of unprecedented fungal outbreaks have emerged, causing serious and often fatal diseases in humans and other animals (Blehert *et al.*, 2009; James *et al.*, 2009; Klein *et al.*, 1986; Rypien *et al.*, 2009). Among the most prominent of these outbreaks is the Vancouver Island outbreak, caused by *Cryptococcus gattii*, which has recently expanded from Vancouver Island (Canada) to the mainland of British Columbia and the Pacific Northwest (Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Kidd *et al.*, 2004).

Until the Vancouver Island outbreak, *C. gattii* was almost exclusively known as the cause of sporadic infections in otherwise healthy humans and animals living in tropical and subtropical regions, mainly in South America and Australasia (Datta *et al.*, 2009; Kidd *et al.*, 2004). However, over the past decade, hundreds of apparently healthy people and animals have been infected by *C. gattii* isolates, all belonging to the AFLP6 lineage, in the affected Vancouver Island outbreak area (Datta *et al.*, 2009). Since the onset of this outbreak, the incidence of *C. gattii* infections on Vancouver Island is estimated to be 25.1 cases per million residents per year, which is approximately 27 times higher than in Northern Australia where *C. gattii* is endemic (Datta *et al.*, 2009; Kidd *et al.*, 2004). Over this period, 19 patients have died due to a *C. gattii* infection, which is a case-fatality rate of 8.7% (Datta *et al.*, 2009). Concurrently with the Vancouver Island outbreak, there have been clusters of AFLP6 *C.gattii* disease in captive psittacine birds in São Paulo, Brazil, (this study; Raso *et al.*, 2004) and in immunocompetent children living in Pará, a province in Northern Brazil (Costa *et al.*, 2009, Santos *et al.*, 2008; Trilles *et al.*, 2008). Finally, a recently performed retrospective study among West Australian veterinary cases has shown that an inexplicable high number of *C. gattii* infections were caused by genotype AFLP6 strains. Strikingly, the prevalence of *C. gattii* AFLP6 was found to be higher in Western Australia compared to the Eastern states of Australia where *C. gattii* was previously found to be endemic (Carriconde *et al.*, 2011; McGill *et al.*, 2009).

All these *C. gattii* outbreaks are caused by genotype AFLP6 strains, which is one of the five known genotypes within this species (AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV) (Bovers *et al.*, 2008; Fraser *et al.*, 2005; Hagen *et al.*, 2010; Kidd *et al.*, 2004; Ngamskulrungroj *et al.*, 2009). AFLP6/VGII can be further differentiated into three subgenotypes, the so-called "major genotype" AFLP6A/VGIIa and the minor genotype AFLP6B/VGIIb and the novel genotype AFLP6C/VGIIc (Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Kidd *et al.*, 2004; Ngamskulrungroj *et al.*, 2009). Comparative virulence studies have suggested that *C. gattii* strains with the major genotype AFLP6A have a higher virulence potential than strains that belong to the minor genotype AFLP6B (Fraser *et al.*, 2005; Ma *et al.*, 2009; Ngamskulrungroj *et al.*, 2010), whilst the recently discovered AFLP6C strains appear to have similar high virulence to that shown by the major genotype AFLP6A strains from the Vancouver Island outbreak (Byrnes *et al.*, 2010).

Eucalyptus trees were initially believed to be the primary ecological niche of *C. gattii*, but extensive environmental sampling revealed that trees in general are the

natural habitat of this yeast (Ellis & Pfeiffer, 1990; Fortes *et al.*, 2001; Grover *et al.*, 2007; Hagen & Boekhout, 2010; Kidd *et al.*, 2004, 2007a, 2007b). Moreover, soil and air in the Vancouver Island outbreak region contain high quantities of *C. gattii* propagules (Kidd *et al.*, 2007b). This may have contributed to the recent range expansion of this organism to the Canadian mainland, which is thought to have been caused by traffic-based transmission of soil and dust-contaminated individuals and motor vehicles (Kidd *et al.*, 2007a).

The molecular epidemiology of the *C. gattii* complex has been studied extensively and led to several hypotheses to explain the origin of the Vancouver Island outbreak (Bovers *et al.*, 2008; Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Kidd *et al.*, 2004; Ngamskulrungroj *et al.*, 2009). Two hypotheses emerged, firstly it has been proposed that same-sex mating between AFLP6B mating-type α strains within the Australasian population, or in transit or in North America, gave rise to a novel highly virulent genotype AFLP6A that subsequently caused the outbreak in Canada (Fraser *et al.*, 2005). In contrast, Ngamskulrungroj *et al.* (2009) observed that the South American population revealed the highest genetic diversity and thus suggested that the origin of the outbreak is most likely in South America.

The origin of this highly virulent lineage within *C. gattii* AFLP6 is strongly debated, but at present remains unclear (Byrnes *et al.*, 2010). To address this, we have undertaken extensive molecular analysis of the *C. gattii* AFLP6 lineage using a combination of Amplified Fragment Length Polymorphisms (AFLP) fingerprinting, a novel multi-locus microsatellite panel and highly discriminative coalescence gene genealogy analysis of SCAR-MLST (Multi-Locus Sequence Typing scheme based on Sequence Characterized AFLP Regions) data. In addition, we have carried out additional sequencing and re-analysis of recently published MLST datasets (Byrnes *et al.*, 2010; Fraser *et al.*, 2005) for which we expanded the geographic range by including more strains from the underrepresented South American population.

These analyses showed that South American *C. gattii* AFLP6 strains represent the ancestral lineages within the global population, in agreement with the observed high genetic diversity at that locality. Coalescence gene genealogy analysis revealed that the basal lineages within genotype AFLP6 have a South American origin, providing strong evidence that South America is the cradle for the global population as well as for the expanding *C. gattii* AFLP6A (Vancouver Island) and AFLP6C (Pacific Northwest) outbreaks. Furthermore, we show that the South American *C. gattii* population has undergone frequent historical recombination, which has been rarely observed for other populations. Thus the hypervirulent *C. gattii* AFLP6 outbreak lineage has likely been derived from a regular mating event in South America rather than a same-sex event in Australia, during transit or in North America. Such a model is further strengthened by the presence of both mating-types in the South American population in almost equal quantities, and by the observation that the hypervirulent trait is present in the most basal South American lineage, represented by an environmental isolate from a tree in the pristine Amazon rainforest.

Material and methods

Strains and media

A global set of 178 *C. gattii* was selected based on previous PCR fingerprinting and AFLP genotype analyses and includes strains involved in the Vancouver Island outbreak (major and minor outbreak lineages AFLP6A and AFLP6B, respectively), Pacific Northwest outbreak AFLP6C, the AFLP6B outbreak among psittacine birds in Brazil and AFLP6B strains from Western Australian that were found to be genetically indistinguishable to those involved in the AFLP6B outbreak at that locality (Byrnes *et al.*, 2010; Carriconde *et al.*, 2011; Kidd *et al.*, 2004; Lindberg *et al.*, 2007). When divided into the five defined populations, the strains came from Africa (*n*=10), Australasia (*n*=38), Europe (*n*=6), North America (*n*=48) and South America (*n*=76) (Supplementary Table 1). Eleven strains, representing all known genotypes, were used as reference strains for molecular studies: WM148 (αA; AFLP1/VNI), WM626Brown and WM626White (αA; AFLP1A/VNII), WM629 (αD; AFLP2/VNIV), WM628 (αA**a**D; AFLP3/VNIII), WM179 (αB; AFLP4/VGI), WM161 (αB; AFLP5/VGIII), WM779 (αC; AFLP7/VGIV), WM276 (αB; AFLP4/VGI), A1M-R265 (αB; AFLP6A) and A1M-R272 (αB; AFLP6B) (Meyer *et al.*, 2009). Strains were cultured and maintained on YPGA-medium (1% w/v yeast-extract, 1% w/v peptone, 2% w/v D-glucose and 2% w/v agar) at 25°C and stored at 4°C on slants or in glycerol-stocks at -196°C.

Intracellular macrophage proliferation assay

A subset of *C. gattii* AFLP6 isolates, indicated in Supplementary Table 1, was tested for their ability to proliferate in J774 macrophages as a measurement of their virulence potential, as described previously by Ma *et al.* (2009).

Mice virulence study

The virulence of the *C. gattii* isolates was studied in a murine model as described by Ma *et al.* (2009). In this model, strains were cultivated at 30°C for 72h on Sabouraud dextrose agar. Yeast cells were suspended in 0.9% w/v saline solution and concentrations were determined by counting with a haemocytometer. A single bolus of a 0.2 ml suspension, containing approximately $2 \cdot 10^6$ cells, was injected into the tail vein of male albino BALB/c mice (weight, 18 - 22 g). Depending on the outcome of the macrophage IPR value, 8 or 9 mice were used for virulence tests when the IPR-value was <1 or ≥1, respectively. Viable count of the yeast cell suspension was measured as the number of CFU on Sabouraud dextrose agar plates after 48h of incubation at 30°C.

Virulence of *C. gattii* strains was based on lethality and determined by recording the number of dead animals in each group daily with a maximum of 45 days. In addition, a daily health evaluation and twice a week weight determination were performed. Statistical analysis was performed using the Kaplan-Meier method in SPSS v18 (SPSS, Chicago, IL). Procedures, care and treatment of the mice were carried in according to the principles of humane treatment outlined by the Guide for the Care and Use of Laboratory Animals of the Hebrew University, approved by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals (approval OPRR-A01–5011).

Analysis of the C. gattii *AFLP6 generation time*

Strains were cultivated for 48 hours on malt-extract agar at 25°C and per strain, one colony was suspended in 100 ml Yeast Nitrogen Base (Difco, BD, Breda, The Netherlands) supplemented with *myo*-inositol (Sigma Aldrich, St. Louis, MO), according to the manufacturer's instructions, and cultured for 48 hours at 25°C under agitation (150 rpm). The carbon source *myo*-inositol, typically assimilated by tremellaceous yeasts, was chosen for the generation experiment since this is an important factor for the sexual development and virulence potential of *Cryptococcus* as well as that it is abundantly present in the human central nervous system (Wang *et al.*, 2011; Xue *et al.*, 2010). Erlenmeyers (300 ml) containing 150 ml Yeast Nitrogen Base (Sigma Aldrich) liquid medium, supplemented with 2% *myo*-inositol, were inoculated with 1.5 ml liquid pre-culture and incubated for 48 hours at 25°C under agitation (150 rpm). Directly after inoculation, 400 µl was used to measure spectrophotometrically the OD_{660}, followed by hourly measurements starting 7 hours after inoculation. Cell concentrations at different time points were counted using a Bürker-Türker counting chamber to determine the increase in cell density.

To calculate the generation time, the natural log of the OD_{660} was plotted against the time (hours) in Microsft Excel 2007 (Microsoft, Redmond, WA). Datapoints before and after the log-growth phase were removed from the curve and the equation ($y = \mu \cdot x + b$) and the R^2 were calculated using a trendline through the remaining data points. The generation time (g) could then be derived when the natural log of µ was calculated, which revealed the number of hours necessary for one *C. gattii* strain to replicate once under the given circumstances. The number of *C. gattii* generations per year is the number of hours per year (8760 hours) divided by g. The generation-time for *C. gattii* per year (n) was determined by cultivating the strains A1M-R265 and CBS1930 in triplicate which resulted in 619 ± 128 and 514 ± 32 generations per year, respectively (Supplementary Figure 8), with an average generation time of 567 ± 93 generations per year.

Ploidy analysis

The ploidy of selected *C. gattii* strains (Supplementary Table 1) was analyzed according to the method described by Bovers *et al.* (2006). The two haploid reference strains A1M-R265 and A1M-R272 and the homozygote diploid *C. gattii* AFLP6A strain RB59JF were included (Fraser *et al.*, 2005). The nuclear content of thirty *C. gattii* AFLP6 strains was investigated using flow cytometry analysis (Supplementary Table 1). All isolates were found to be haploid, compared to the two reference strains and the diploid strain RB59JF. An independent sub-culture of the isolate RB59 (designated RB59FH in the current study) was found to be haploid whereas another sub-culture was confirmed to be diploid (designated in the current study as RB59JF) as originally reported and received directly from another research group (Fraser *et al.*, 2005). Another sub-culture of this isolate (RB59WM), which was sent from the original distributor at the same time to a third research group, was subsequently found to be haploid. The most parsimonious explanation is that the sub-culture of RB59JF became diploid via either autodiploidization, same-sex mating, or cell-cell fusion.

DNA extraction
Genomic DNA was extracted as described previously (Hagen *et al.*, 2010). Briefly, cultures were allowed to grow for 48h at 25°C on YPGA-medium supplemented with 0.5 M sodium chloride to prevent capsule formation. Approximately 150 µl of cells were harvested and dissolved in 1.6 ml urea buffer and incubated at room temperature for 3 hours. Cells were lysed by bead beating at 2500 rpm for 3 minutes in phenol-chloroform-isoamylalcohol (25:24:1; pH 8.0). Genomic DNA was precipitated with ice-cold EtOH 96% and 100µl ammonium acetate 3.0 M and afterwards dissolved in 100 µl Tris-EDTA buffer (pH 8.0). Genomic DNA was purified using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ).

Mating-type determination
Partial amplification of the *STE12* gene, as described by Bovers *et al.* (2006), was performed to determine mating-type **a** and α specific regions of all strains using *C. gattii* isolates CBS1930 (**a**B) and CBS6956 (αB) as reference strains.

AFLP fingerprinting
Amplified Fragment Length Polymorphism (AFLP) analysis was carried out in duplicate on an ABI Prism 310 Genetic Analyzer platform (Applied Biosystems, Foster City, CA) as described previously (Boekhout *et al.*, 2001) using the following four combinations of selective primers: *Eco*R1-AC/*Mse*I-G, *Eco*R1-AC/*Mse*I-C, *Eco*R1-AG/*Mse*I-G and *Eco*R1-AT/*Mse*I-G. A set of five *C. neoformans* (WM148, WM626Brown, WM626White, WM628 and WM629) and four *C. gattii* strains (WM161, WM178, WM179 and WM779) were used as a reference (Supplementary Table 1). The presence and absence of specific loci in the four duplicated AFLP patterns were scored manually, according to Campbell *et al.* (2005). The distance between isolates was calculated with PAUP v4.0.10b (Sinauer Associates, Sunderland, MA) using the Neighbour Joining (BioNJ method) algorithm. For this analysis, ties were broken randomly and, when characters were observed to be different, the 'mean character difference' was used. The restriction-site distance was calculated using uphold and with 'among site variation'.

Multi-Locus Sequence Typing approaches
The Sequence Characterized AFLP Regions Multi-Locus Sequence Typing (SCAR-MLST) scheme was developed based on sequenced polymorphic markers obtained by differential Amplified Fragment Length Polymorphism (dAFLP) fingerprinting analysis (Xu *et al.*, 2001). Twelve strains, indicated in Supplementary Table 1, were selected from the phylogenetic analysis based on the AFLP matrix described above. Fragments were excised from the polyacrylamide gel and the selective primers from the initial AFLP selective PCR were used to generate amplicons from these excised fragments. The amplicons were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ), followed by agarose gel electrophoresis to check quantity and quality.

Sequencing of the excised AFLP fragments was carried out using the BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) using unlabeled primers

identical to those used for the selective AFLP PCR. Sequencing reactions were carried out as described by Bovers *et al.* (2008). Raw data was automatically aligned using SeqMan v5.03 (DNASTAR, Madison, WI) and ambiguously placed nucleotides were manually corrected.

AFLP fragment consensus sequences were compared to the available genomes of *C. gattii* strain A1M-R265 (serotype B; Broad Institute; http://www.broad.mit.edu/; D'Souza *et al.*, 2011) and the annotated genome of *C. neoformans* var. *neoformans* strains JEC21 and B3501 (serotype D; TIGR; http://www.tigr.org/; and The Stanford Genome Technology Center; http://www-sequence.stanford.edu/, respectively; Loftus *et al.*, 2005) using a local BLAST approach (ftp://ftp.ncbi.nlm.nih.gov/).

Fragments that corresponded to known functional categories of genes, or that had high homology with predicted proteins, were used as starting-point for primer design using GeneFisher (Giegerich *et al.*, 1996). Primers were designed, based on the genome of *C. gattii* strain A1M-R265, at the end of the approximately 350 nucleotides long flanking regions of the sequenced original fragment, with a maximum length of 25 nucleotides and a melting temperature between 50 to 65°C (Supplementary Table 2). A subset of twelve *C. gattii* isolates, indicated in Supplementary Table 1, was used to amplify the obtained polymorphic markers and thereby identifying the fragments with the highest proportion of polymorphic sites. Amplicons were sequenced and analyzed as described above, resulted in the SCAR-MLST (Sequence Characterized AFLP Regions - Multi-Locus Sequence Typing) dataset that comprised five nuclear loci and one mitochondrial locus. Sequence type numbers were assigned using the non-redundant database (http://www.mlst.net). Sequences were deposited in Genbank with the accession numbers HM997344-HM998285 and HQ706505-HQ706637.

Additionally, a set of 36 strains (Supplementary Table 1) were used to supplement the MLST dataset of Fraser *et al.* (2005) and Byrnes *et al.* (2010). The *MPD1*-locus was excluded because it contained no polymorphic sites among the AFLP6 strains (Fraser *et al.*, 2005). Additional strains came from Europe (n=4) and South America (n=32). Amplification of these loci was performed as described by Fraser *et al.* (2005), sequence analysis was carried out as described above. Sequence type numbers were assigned according to those published by Fraser *et al.* (2005) and Byrnes *et al.* (2010) and new ST numbers were assigned to novel sequence types as detected by the non-redundant database software (http://www.mlst.net). In the current study, we refer to this dataset as the extended 'Fraser and Byrnes' MLST dataset to distinguish it from the above developed SCAR-MLST dataset.

Microsatellite analysis

Microsatellite typing involved a panel of ten microsatellite markers consisting of six di-nucleotide repeat markers, one tri-nucleotide repeat markers and three hexa-nucleotide repeat markers. Primer sequences are indicated in Supplementary Table 2. All markers were amplified in four multicolor multiplex amplification reactions (containing three markers each, except for the tri-nucleotide-repeat marker). Amplification was performed by applying an initial denaturation step for 10 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 60°C and 60 s at 72°C. An additional incubation for 10 min at

72°C was applied before the reactions were cooled down. Reaction mixtures contained 1 U FastStart Taq DNA polymerase (Roche Diagnostics, Almere, The Netherlands), 1 ng DNA, 10 pmol of each primer, 0.2 mM dNTP's and 3 mM $MgCl_2$ in a total volume of 50 µl of 1× PCR reaction buffer. Following amplification, PCR products were analyzed on a MegaBACE™ 500 platform (GE Healthcare, Diegem, Belgium) equipped with a 48 capillary array as described by Illnait-Zaragozí *et al.* (2010). Repeat numbers were determined by comparing the relative size of the obtained fragments to those obtained using the reference strain A1M-R265 whose genome has been elucidated. Microsatellite repeat numbers are listed in Supplementary Table 1.

Occasionally, non-integer alleles were obtained as the result of the presence of indels in the flanking region of the repeat region. The identity of the indels and verification of repeat numbers in selected alleles was performed by subcloning amplicons in a pJET1.2 cloning vector (Fermentas, St. Leon-Rot, Germany) followed by direct sequencing, as described above, of the microsatellite insertion from the purified plasmid DNA. Repeat numbers in non-integer alleles were rounded off to the appropriate integer according to the identity of the indel.

A minimum spanning tree was generated with BioNumerics v4.6 (Applied Maths, Sint-Martens-Latem, Belgium) using a categorical analysis and UPGMA clustering. Microsatellite complexes (MCs) were determined to have a minimum size of two types and with a maximum neighbor distance of two loci among the ten studied.

Phylogenetic sequence analysis

Multiple sequence alignments were generated in MEGA v5 (Tamura *et al.*, 2011) with the ClustalW algorithm. Loci were concatenated using FSorter, a script written and executable in Python (http://www.python.org/). For phylogenetic analysis, the best fitting nucleotide substitution model was determined using MrModeltest v3.7 (http://www.abc.se/~nylander/), optimal nucleotide substitution models observed are listed in Supplementary Table 2. MEGA v5 (Tamura *et al.*, 2011) was used to perform a bootstrapped Maximum Likelihood phylogenetic analyses with 1000 iterations using the optimal nucleotide substitution of Hasegawa-Kishino-Yano model with invariant sites and gamma distributed, which was found to be the best fitted model for the concatenated nuclear SCAR-MLST dataset.

Genetic diversity

Simpson's diversity index (*D*) was used to determine the discriminatory power of the AFLP, microsatellite and MLST data, as well as to assess the genetic diversity among the defined populations (Simpson, 1949).

Recombination analysis using DnaSP

To calculate the minimum number of recombination events (R_M) the software package DnaSP v5.1 was used (Hudson, 1987; Hudson & Kaplan, 1985; Librado & Rozas, 2009). Recombination events were calculated as the minimum number of recombination events between two adjacent sites derived from the 'four gametes test', however, this is an underestimated number of recombination events (Hudson, 1987; Hudson &

Kaplan, 1985). DnaSP v5.1 was also used to calculate the Hudson-parameter 'Hud4Nc per site' according to Hudson (1987). Next to these recombination tests, the nucleotide diversity per site (π) corresponding to the average number of nucleotide differences per site between two sequences, the number of segregating, or polymorphic, sites (S), the Watterson's estimated θ per locus and per nucleotide (θ=4Nm, with N being the effective population size and m the mutation rate per nucleotide per generation) were calculated using DnaSP v5.1. Results are listed in Supplementary Table 5.

Recombination analysis using the Pairwise Homoplasy Index

The Pairwise Homoplasy Index, or Phi (Φ_W) test was used to distinguish recurrent mutations from recombination and was calculated for the complete concatenated datasets of the nuclear SCAR-MLST loci and the extended Fraser MLST (SplitsTree v4.7.18; Bruen *et al.*, 2006).

Recombination analysis using the CASS algorithm

A reticulated network of the SCAR-MLST was calculated using the recently developed algorithm CASS (Van Iersel *et al.*, 2010). The five nuclear loci from the SCAR-MLST were separately analyzed and for each locus an unrooted phylogenetic tree was constructed using the implementation of the Neighbour Joining algorithm available in the SplitsTree v4.7.18 package (Huson & Bryant, 2006). In each case the tree was rooted with strain LMM645 as this was the most basal lineage based on the coalescence gene genealogy. We calculated bootstrap values for each gene tree using 2000 bootstrap replicates. Subsequently, we selected those clades that had bootstrap support of at least 90%. Nine clades fulfilled this property. A phylogenetic network representing all these clades was constructed using the CASS algorithm, which is available as part of the software package Dendroscope v2 (Huson *et al.*, 2007).

Ancestral recombination graph

To reconstruct the Ancestral Recombination Graph (ARG) of *C. gattii*, the sequence data from the complete set of five SCAR-MLST markers was concatenated. Beagle was used to compute the minimum number of recombination events (Lyngsø *et al.*, 2005) and to simultaneously reconstruct a minimal ancestral recombination graph (ARG) using the Kwarg branch and bound algorithm implemented in Beagle (Lyngsø *et al.*, 2005). Kwarg implements a heuristic search, reducing computation time without significant loss of power (Lyngsø *et al.*, 2005). The reconstructed ARG is displayed in a hierarchical-direct graph, and can be rooted with the most recent common ancestor (MRCA) of the entire sample, inferred from coalescent simulations. The rooted ARG provides inferences on the relative order of recombination events and the contribution of mutation and recombination in the evolution of haplotypes.

Inferring the coalescent history of mutations: neutrality tests, population subdivision analysis, migration and coalescence

In order to reconstruct the history of the mutations in our data set, we used coalescent methods, which have strict assumptions, such as neutrality and absence of recombination signals (identified as incompatible homoplasious sites). Sixty-eight incompatible homoplasious sites were manually removed from the concatenated nuclear SCAR-MLST alignment, producing a dataset with no incompatibilities and 109 informative sites remained, the removal of sites caused a 35% reduction in haplotypes from 49 to 32. Neutrality tests were performed in DnaSP v5.1 (Librado & Rozas, 2009), Tajima's *D* (Tajima, 1989), Fu and Li's *D* and *F* (Fu & Li, 1993) were calculated. Deviations from neutrality could be a sign of selection or changes in the effective population sizes, such as population expansion or contraction. Analyses were performed for every locus as well as for each population independently. Neutral evolution for the nuclear loci was observed except for the IGS1 locus ($P<0.05$).

Coalescence gene genealogy analysis

The coalescent gene genealogy was simulated under the scenario of population subdivision and distinct population sizes using SNAP Workbench v2 (Price & Carbone, 2005). Historical migration matrices and θ values for the coalescent simulations were estimated previously with Migrate v3.1.6 (Supplementary Table 4) (Beerli & Felsenstein 2001). Estimates were obtained using two hundred independent replicate runs, each run with ten initial short chains, two final long chains and static heating schemes with four temperatures (1.0, 1.3, 2.6 and 3.9), swapping interval of one. Confidence intervals for θ and *M* were calculated using a percentile approach (Beerli & Felsenstein 2001). Subsequently, we reconstructed coalescent SCAR-MLST haplotype genealogies containing the ages of mutations backwards in time and the time for the most recent common ancestor (TMRCA) using Genetree v9.0 (Bahlo & Griffiths, 2000; Griffiths & Tavaré, 1994). Ten initial simulations were performed, starting from ten distinct random seed numbers, corresponding to 10 million runs each. The coalescent haplotype genealogies with the highest likelihood were chosen from the initial ten simulations and a second simulation with ten million iterations was performed.

The topology of the coalescent genealogy with time scale in coalescent units was constructed and depicted using Genetree/Treepic (Bahlo & Griffiths, 2000; Griffiths & Tavaré, 1994). Ages of mutations in real time scale were generated as follows: the coalescence time t in years was calculated as $t=t/\mu \cdot n$, where *t* is the Genetree TMRCA estimate, μ is the overall mutation rate for *C. gattii* ($2 \cdot 10^{-9}$ substitutions per nucleotide per year; D'Souza *et al.*, 2011; Sharpton *et al.*, 2008) and *n* is the generation time of *C. gattii*. The generation time *n* for *C. gattii* was determined using *myo*-inositol as the sole carbon source for strain A1M-R265 (H1; AFLP6A, mating-type α) and CBS1930 (H19; AFLP6B, mating-type **a**) were 619 ± 128 and 514 ± 32 generations per year, respectively, and were not significantly different ($P=0.281$).

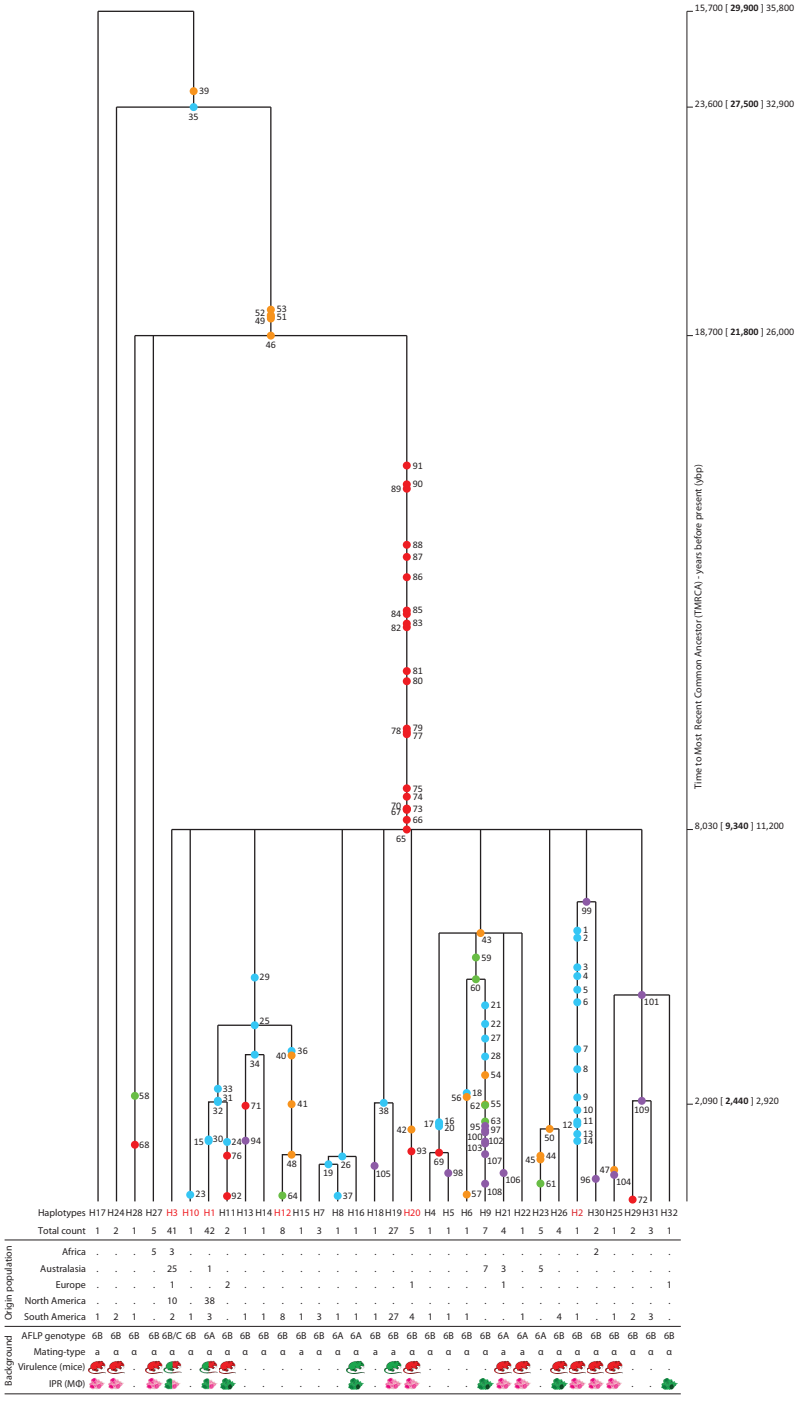
Results

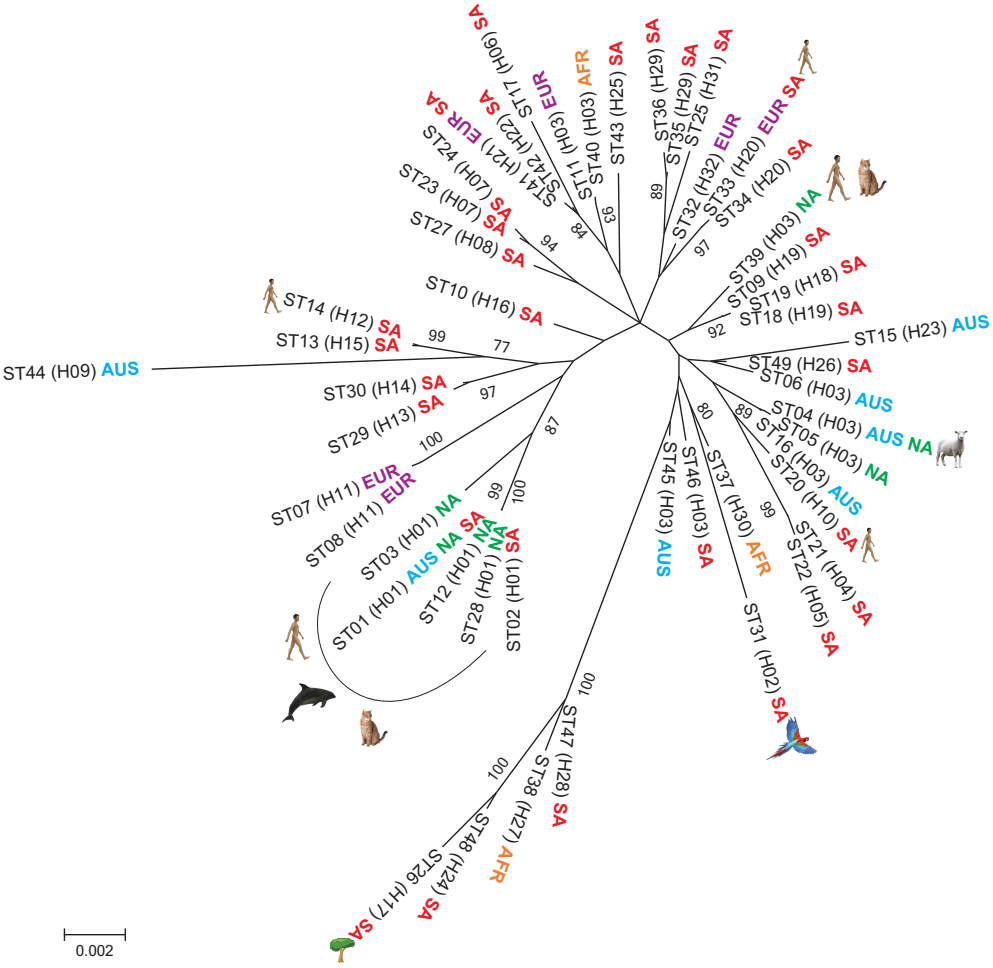
South America is the origin for the global* C. gattii *AFLP6 population

The most basal lineage in the coalescence gene genealogy was found to be of South American (SA) descent, represented by an environmental strain from pristine Brazilian Amazon rainforest (LMM645; H17, AFLP6B, mating-type **a**), followed by the SA lineage H24 (LMM265 and LMM266; Brazil, AFLP6B, mating-type α) (Figure 1). Mutations deep in the coalescence gene genealogy divided the two basal lineages from three younger branches, which were differentiated from each other by more recent mutations. Two of these ancient lineages had an African (AFR) and SA origin (H27 and H28) while the third lineage subsequently further diverged over time into 28 haplotypes after the occurrence of 22 mutations in the 'Fragment 54' locus. Compared to the other outbreak lineages, the psittacine bird outbreak lineage H2 contains substantially more mutations due to a 20bp region in the non-coding locus 'Fragment 15' which is highly polymorphic compared to the other four nuclear SCAR-MLST loci (Figure 1; Supplementary Table 3).

Time scaled coalescence analysis revealed that the major clade (H1-H16, H18-H23 and H25-H32) and the basal clade (H17 and H24) had a TMRCA approximately 30,000 years before present (ybp) (Figure 1). The lineage representing the major genotype Vancouver Island outbreak lineage H1 emerged approximately 2,500 ybp. The other outbreak lineages, representing the *C. gattii* outbreak among psittacine birds (H2) emerged 7,500 ybp, while lineages involved in cryptococcosis among children (H10, H12 and H20) appeared 9,300 and approximately 1000 ybp. Interestingly, a major diversification event occurred approximately 9,300 years ago that also gave rise to a global lineage (H3) that includes strains from the *C. gattii* AFLP6C outbreak in the Pacific Northwest and strains from the Vancouver Island outbreak (AFLP6B). The latter are indistinguishable to the outbreak isolates among over 100 sheep in Western Australia based on multi-locus sequence typing (Carriconde *et al.*, 2011) (Figure 1).

Next page, Figure 1. The coalescence gene genealogy of the global *C. gattii* AFLP6 population structure was reconstructed using five nuclear SCAR-MLST loci that included 109 informative sites indicated on the branches as blue ('Fragment 15' locus), orange ('Fragment 32' locus), green ('Fragment 34' locus), red ('Fragment 54' locus) and purple (IGS1 locus) dots. Numbers next to these dots represents the position of the site within the sequence of informative sites as indicate in Supplementary Table 3. Haplotypes that represent outbreak lineages are coloured red, with H1 as the major genotype AFLP6A Vancouver Island outbreak, H2 the outbreak among psittacine birds in Brazil, H3 the minor genotype AFLP6B Vancouver Island outbreak as well as the genotype AFLP6C outbreak in the Pacific Northwest (USA) and the outbreak among sheep in Western Australia, and the lineages H10, H12 and H20 representing lineages involved in *C. gattii* infections among children in Para (Brazil) and French Guiana. Numbers indicated behind the population names are the number of strains within the given population and haplotypes. Background information of the strains per genotype is given for the AFLP genotype, mating-type, and virulence in mice and the intracellular proliferation rate inside J774 macrophages. The latter two assays are given as red or green mice and macrophages representing respectively hypervirulent and non-virulent strains as indicated in Figure 4 and Supplementary Table 1. The TMRCA in 'years before present' is provided, including the lower and upper boundaries (bold), as an indication of the divergence time for the major clades as well as for the major genotype AFLP6A Vancouver Island outbreak.


15,700 [29,900] 35,800
23,600 [27,500] 32,900
18,700 [21,800] 26,000
8,030 [9,340] 11,200
2,090 [2,440] 2,920
Time to Most Recent Common Ancestor (TMRCA) - years before present (ybp)
Haplotypes H17 H24 H28 H27 H3 H10 H1 H11 H13 H14 H12 H15 H7 H8 H16 H18 H19 H20 H4 H5 H6 H9 H21 H22 H23 H26 H2 H30 H25 H29 H31 H32
Total count 1 2 1 5 41 1 42 2 1 1 8 1 3 1 1 1 27 5 1 1 1 7 4 1 5 4 1 2 1 2 3 1
Origin population
Africa . . . 5 3 . . . . 2 . . . .
Australasia . . . . 25 . 1 . . . . 7 3 . 5 . . . . . .
Europe . . . . 1 . . 2 . . . . 1 . . . . . 1 1
North America . . . . 10 . 38 . . . .
South America 1 2 1 . 2 1 3 . 1 1 8 1 3 1 1 1 27 4 1 1 1 . . 1 . 4 1 . 1 2 3 .
Background
AFLP genotype 6B 6B 6B 6B 6B/C 6B 6A 6B 6B 6B 6B 6B 6B 6A 6A 6B 6B 6B 6B 6B 6B 6B 6A 6A 6B 6B 6B 6B 6B 6B 6B 6B
Mating-type a α α α α α α α α α a α α α α a α α α α α α α α α α α α α
Virulence (mice)
IPR (MΦ)



Above, Figure 2. Unrooted Maximum Likelihood phylogenetic analysis of the sequence types found within the concatenated dataset of five nuclear SCAR-MLST loci. The STs corresponding to haplotypes H17, H24, H27 and H28, being the ancestral lineages in the coalescence analysis, were found to be the most basal lineages when rooted with *C. gattii* AFLP4 (data not shown). The most ancestral lineage H17 is indicated by a tree to highlight its environmental origin from pristine Amazon rainforest. STs that fell into H1 as the major genotype AFLP6A Vancouver Island outbreak lineage, H2 as the outbreak among psittacine birds in Brazil, H3 the minor genotype Vancouver Island outbreak lineage (ST4 and ST5), the recently emerged genotype AFLP6C outbreak in the Pacific Northwest (ST39) and the outbreak among sheep in Western Australia (ST4) are indicated with symbols referring to its origin (e.g. human referring to human infections, sheep from the outbreak among sheep, cat representing veterinary cases, dolphin representing infections among sea animals). Bootstrap values are given for branches that are highly supported (≥75).

High virulence is not restricted to C. gattii *strains within the outbreak genotype AFLP6A*

Previous studies have shown that *C. gattii* AFLP6A and AFLP6C outbreak strains are highly virulent in different host models, while strains that belong to genotype AFLP6B were found to be less virulent or even avirulent (Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Ma *et al.*, 2009; Ngamskulrungroj *et al.*, 2010). Strikingly, it was observed that the environmental strain CBS7750 from California and the clinical strain CBS6956 (= NIH444) from Seattle were avirulent or had attenuated virulence, respectively, while Vancouver Island outbreak strains occurring in the same lineage were found to be highly virulent (Byrnes *et al.*, 2010; Ma *et al.*, 2009; Ngamskulrungroj *et al.*, 2009).

To further elucidate the virulence potential among strains within the more basal lineages in the coalescence tree, we investigated *C. gattii* isolates using mice and macrophage pathogenicity assays (Byrnes *et al.*, 2010; Ma *et al.*, 2009; Figure 1 and 3; Supplementary Table 1). This revealed that the ancestral coalescence lineages represented by the SA strains LMM645 (H18; AFLP6B, mating-type **a**) and LMM265 (H24; AFLP6B, mating-type α) were highly virulent (ST_{50} = 10.0 ± 0.0 days each). Despite the observation that these two strains had the highest virulence potential among genotype AFLP6B strains, it was found that they were significant less virulent than the hypervirulent *C. gattii* AFLP6A strains from the Vancouver Island outbreak region which had ST_{50} values ranging from 3.9 ± 0.1 to 6.8 ± 0.3 days (Figure 3; Supplementary Table 1). However, the macrophage intracellular proliferation rates (IPR) of these two SA strains were among the highest observed, with values of 2.54 for LMM265 and 2.37 for LMM645 (Byrnes *et al.*, 2010; Ma *et al.*, 2009; Supplementary Table 1). The highest IPR values were observed for the three AFLP6B mating-type α strains IP2001/935-1 (H27; AFR), IP1997/170 (H25; SA) and IP2004/335 (H20; SA) which had IPR values of 2.92, 2.82 and 2.75, respectively. These high IPR values were reflected by their high pathogenicity in BALB/c mice (Figure 3; Supplementary Table 1). Interestingly, however, within this diverse group a high IPR value does not necessarily predict a similar high virulent phenotype in the BALB/c mice model (Figure 3; Supplementary Table 1).

Genetic diversity and phylogenetic analyses reveal a centre of diversity in South America

Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis, Sequence Characterized AFLP Regions-based Multi-Locus Sequence Typing (SCAR-MLST) and microsatellite analysis (Figure 2; Supplementary Figure 1 and 2) was used to assess the genetic diversity of *C. gattii* genotype AFLP6. The Simpsons diversity index revealed that microsatellite typing showed the highest discriminatory power (*D*=0.940), followed by AFLP (*D*=0.928) and SCAR-MLST (*D*=0.909). The combined AFLP, SCAR-MLST and microsatellite data showed that the AFR and European (EUR) populations had the highest diversity with *D*-values of 0.978 and 1.000, respectively. However, it is likely that the effective diversity in SA is higher than in AFR and EUR since the effective population sizes of the latter populations are relatively small compared to SA (Figure 4; Supplementary Table 4). In addition, AFR and EUR populations contain solely clinical strains that might be travel-related infections while the Australasian (AUS),

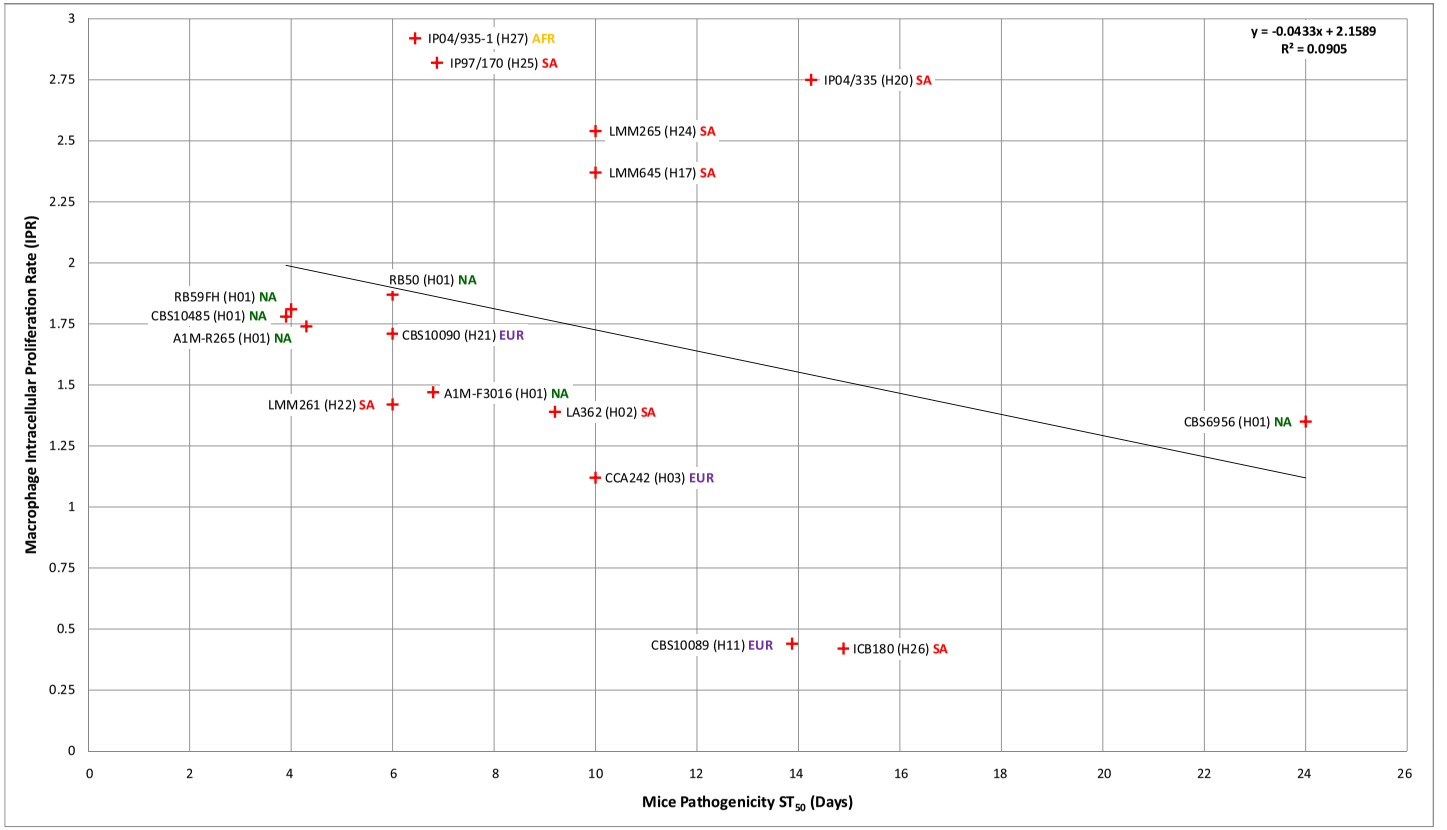


Figure 3. Pathogenicity based on macrophage J774 Intracellular Proliferation Rate (IPR) and ST_{50} in BALB/c mice. The fifty percent survival time (ST_{50}) in days for BALB/c mice infected with *C. gattii* is presented on the x-axis and plotted against the IPR of the same *C. gattii* strain inside murine J774 macrophage cells. Strains that were not virulent in the BALB/c mice experiment, with a maximum of 45 days of monitoring, were excluded from this graph. Excluded strains with a non-virulent phenotype for the BALB/c mice pathogenicity assays were A1M-R272, CBS1930, CBS7750, CBS8684 and IHEM11489. For detailed IPR and ST_{50} survival values see Supplementary Table 1.

Table 1. Genetic diversity of the different *C. gattii* populations based on AFLP, SCAR-MLST and Microsatellite typing.

Population	differential AFLP		SCAR-MLST nuclear loci		SCAR-MLST all loci		Microsatellite typing		Combined	
	$n_{GENOTYPES}$	*D*	$n_{GENOTYPES}$	*D*	$n_{GENOTYPES}$	*D*	$n_{GENOTYPES}$	*D*	$n_{GENOTYPES}$	*D*
Africa (*n*=10)	5	0.822	3	0.689	4	0.733	6	0.844	9	0.978
Australasia (*n*=38)	8	0.664	7	0.627	8	0.657	15	0.765	19	0.835
Europe (*n*=6)	5	0.933	6	1.000	6	1.000	5	0.933	6	1.000
North America (*n*=48)	6	0.634	6	0.455	6	0.455	12	0.645	18	0.837
South America (*n*=76)	33	0.877	31	0.870	34	0.871	37	0.929	48	0.951
All (*n*=178)	52	0.928	49	0.907	53	0.909	72	0.940	97	0.969

The genetic diversity, based on Simpsons diversity index (*D*) for each of the defined populations, as well as for all *C. gattii* AFLP6 strains, is shown. Differential AFLP refers to the matrix of arbitrary scored AFLP markers. Diversity of the SCAR-MLST has been given for all six loci as well as for solely the five nuclear loci. Diversity values range 0.000 to 1.000, the higher the *D* value, the higher the genetic diversity is.

North American (NA) and SA populations are a mixture of clinical, veterinary and environmental strains. Thus SA has the highest genetic diversity with 48 genotypes among 76 isolates (*D*=0.951) suggesting that this population is the origin of the global *C. gattii* AFLP6 population. In support of this, all of the genotyping methods (AFLP, SCAR-MLST and microsatellite typing) indicate that SA has the highest diversity compared to AUS and NA (Table 1).

Phylogenetic analyses indicate that C. gattii *AFLP6 outbreaks have a South American origin*

Maximum Likelihood phylogenetic analysis using the nuclear SCAR-MLST loci revealed that a highly supported basal lineage contained strains from AFR and SA (Figure 2). All basal AFR strains were AFLP6B mating-type α, while both mating-types were present among the SA AFLP6B lineages. From this basal cluster, other well supported lineages arose, including three major clades that originate from SA (AFLP6B, mating-type **a**), a mixed clade of AUS and NA isolates (AFLP6B, mating-type α) and a group of Vancouver Island outbreak (NA) isolates (AFLP6A, mating-type α) with a diverse basal group of AFR, AUS, EUR and SA isolates that exhibit different AFLP6 subgenotypes and mating-types. The Vancouver Island outbreak cluster contains only *C. gattii* genotype AFLP6A isolates, including three isolates that originated from localities in Thailand (MC-S-239), Argentina (LA295) and Brazil (IFM47258). This phylogenetic analysis showed that the recently emerged Pacific Northwest outbreak lineage AFLP6C is distantly related to the expanding Vancouver Island outbreak genotype AFLP6A. The *C. gattii* AFLP6B outbreak among psittacine birds seems to

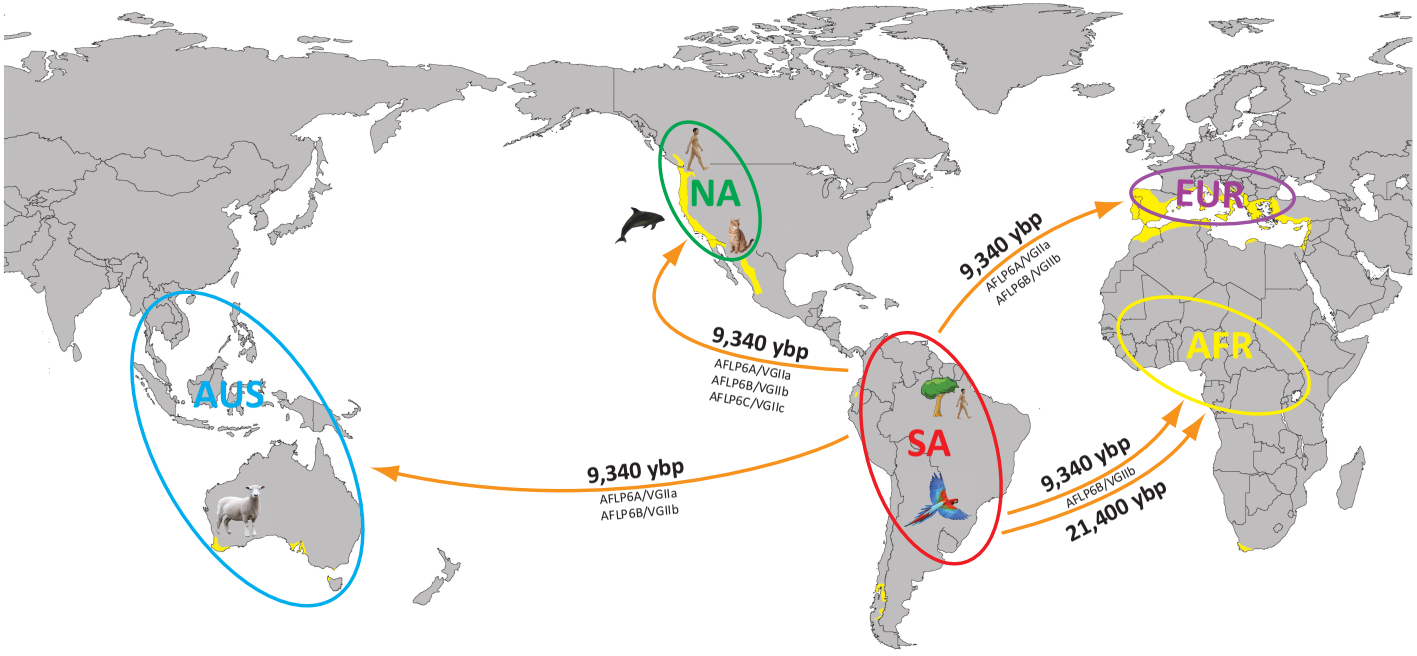


Figure 4. Proposed model of ancient *C. gattii* dispersal and population structure. The *C. gattii* AFLP6/VGII populations Africa (yellow), Australasia (blue), Europe (purple), North America (green) and South America (red) are indicated by similar coloured ellipses. Orange coloured arrows indicated the major migration events from the oldest population (South America, specifically the Amazon rainforest in Brazil) with the estimated time frame given in years before present (ybp). The approximate geographical location of the most basal lineage of coalescence gene genealogy (H17) is indicated with a tree. The outbreaks in North America are indicated with icons of human, cat and dolphin, the outbreak among sheep in Western Australia is indicated with a sheep, and the human and parrot in the South American population highlight the high proportion of children developing disease caused by *C. gattii* AFLP6, as well as the outbreak of *C. gattii* among psittacine birds in Southern Brazil. Yellow coloured areas in the map represent localities with a Mediterranean sea climate according to the updated Köppen-Geigen classification (adapted from Peel *et al.*, 2007).

be non-related to any of the other outbreaks, while the outbreak among sheep in Western Australia was recently found to be indistinguishable from strains involved in the outbreak at Vancouver Island, i.e. the so-called minor genotype AFLP6B lineage, based on MLST data (Carriconde *et al.*, 2011; Figure 2).

The mitochondrial derived SCAR-MLST locus "Fragment 23", that partly encodes for the cytochrome c oxidase gene *COX2*, revealed that strains from the Vancouver Island outbreak had a *COX2* locus similar to SA *C. gattii* AFLP6A and AFLP6B lineages (H1, H3, H8, H12, H15, H21, H26 and H28). One of these SA lineages represents a basal lineage in the coalescence gene genealogy (H28; Colombian isolate H0058-I-1543). The *C. gattii* AFLP6C outbreak lineage (H3) was found to have a *COX2* locus similar to the strain involved in the *C. gattii* AFLP6B outbreak among psittacine birds in Southern Brazil (LA362; H2). Strains with the Vancouver Island outbreak minor genotype AFLP6B that were previously shown to be identical to strains involved in the *C. gattii* AFLP6 outbreak among sheep in Western Australia, were found to cluster together with strains from AUS, EUR and SA (Supplementary Table 1; Supplementary Figure 6).

To further support our hypothesis that the *C. gattii* AFLP6 Vancouver Island outbreak arose from a non-Australian population, we sequenced the nuclear loci *CAP10*, *GPD1*, IGS1, *LAC1*, *PLB1* and *TEF1α* for 36 isolates that originated from SA (*n*=32) and EUR (*n*=4), to complement the MLST datasets generated by Fraser *et al.* (2005) and Byrnes *et al.* (2010) in which the SA population was underrepresented (Supplementary Table 1; Genbank accession numbers HM990672-HM990953). A total of 135 *C. gattii* isolates were used to re-analyze the 'Fraser & Byrnes' MLST dataset (Supplementary Figure 5). This analysis revealed that several clades with AUS, NA and SA strains emerged from the basal SA cluster. The large cluster of *C. gattii* AFLP6A Vancouver Island outbreak strains emerged from a basal lineage with AUS strains.

We additionally investigated the coherence between the global *C. gattii* AFLP6 strains using an AFLP derived matrix and a novel microsatellite panel. These data analyses revealed additional evidence that the recent emergence of *C. gattii* AFLP6C in the Pacific Northwest was caused by a genetically distinct lineage that could not be linked to the ongoing Vancouver Island *C. gattii* AFLP6A outbreak (Supplementary Figure 1 and 2).

Recombination occurs in all C. gattii *AFLP6 populations and is the highest among South American isolates*

Despite extensive sampling, no mating-type **a** strains were found in Africa and North America, and only recently mating-type **a** strains were found in Australia (Carriconde *et al.*, 2011; Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Hagen *et al.*, 2010; Kidd *et al.*, 2004). Several epidemiological studies found that both mating-type **a** and α strains are present within the European and South American *C. gattii* AFLP6 populations (Carriconde *et al.*, 2011; Escandón *et al.*, 2006; Hagen *et al.*, 2010; Trilles *et al.*, 2008; Ngamskulrungroj *et al.*, 2009). *STE12* mating-type analysis of the 178 *C. gattii* AFLP6 strains used for the current study confirmed that the EUR

and SA populations contained both mating-type **a** and α strains (Supplementary Table 1).

The presence of 34 mating-type **a** and 42 mating-type α strains in the SA population suggest that recombination events occur in this population, however, it has been shown that the South American mating-type **a** strains are less fertile than their mating-type α counterparts from SA and the Vancouver Island outbreak (Ngamskulrungroj *et al.*, 2008). Recombination has also been observed within exclusively mating-type α populations (Campbell *et al.*, 2005; Saul *et al.*, 2008) and it has been demonstrated that same-sex exists as a mode of reproduction for *C. gattii* and its sibling *C. neoformans* (Fraser *et al.*, 2005; Lin *et al.*, 2005, 2007, 2009).

The full-length nuclear SCAR-MLST sequences were used to search for evidence of recombination using Ancestral Recombination Graph (ARG). The ARG analysis showed the presence of extensive recombination in the history of the global set of *C. gattii* AFLP6 isolates, as illustrated by 167 historical recombination events (Supplementary Figure 4). The level of recombination observed differed widely per population. The number of recombination events was disproportionally high in SA (110 events), whereas that in the AUS, EUR and NA population was low (8, 5 and 1 events, respectively) and undetectable in AFR (Supplementary Figure 4). Additionally, three recombination events were observed in the mitochondrial *COX2* locus ('Fragment 23') using ARG (Supplementary Figure 7).

Recombination was also observed with tests that calculated the minimum number of recombination events (R_M) which is derived from the 'four gametes test'. Both tests resulted in traceable recombination events in the AUS, EUR and SA populations. The latter was found to contain more recombination events than those in AUS and EUR (47 versus 2 and 1) (Supplementary Table 5).

The Φ_W test found statistically significant evidence for recombination in the SCAR-MLST (P<0.001) but not within the extended 'Fraser and Byrnes' MLST set (P=0.196). When the SCAR-MLST data was split into the five populations, evidence for recombination was found in the AUS (P=0.033), EUR (P=0.044), NA (P<0.001) and SA (P<0.001) populations. No recombination was observed in the mitochondrial *COX2* locus ('Fragment 23') when the Φ_W test was applied (P=0.120).

CASS network analysis only revealed highly supported signs of recombination in the SCAR-MLST dataset. In this analysis, the basal lineages were similar to those observed in the coalescence and phylogenetic approaches. Here, a small cluster with AUS strains (H23) was found to be a recombinant between the precursors of the basal SA strain LMM645 (H17) and lineage H7. Two large clades, that include the Vancouver Island outbreak lineage H1 as well as a large cluster with genotype AFLP6B isolates, were a result of ancient recombination between a precursor from within the basal SA lineages with either the SA lineage H7 or a precursor of the recombinant strain ENV133 (H1; Vancouver Island outbreak) (Supplementary Figure 3).

Discussion

Fungal outbreaks have emerged over recent decades and affected humans, mammals, reptiles and insects. These outbreaks are caused by previously rare genotypes or novel species, such as white-nose-syndrome in bats (*Geomyces destructans*), the frog-killing fungus *Batrachochytrium dendrobatidis*, honeybee colony collapses (*Nosema ceranae*) and the ongoing *Cryptococcus gattii* outbreaks in Vancouver Island (Canada), the Pacific Northwest, Western Australia and Brazil (Blehert *et al.*, 2009; Byrnes *et al.*, 2010; Carriconde *et al.*, 2011; Higes *et al.*, 2008; James *et al.*, 2009; Kidd *et al.*, 2004; Raso *et al.*, 2004). Understanding the genetic and geographic origin of these outbreaks is critical in order to limit the spread of infection. Here we provide a model for the origin of the *C. gattii* epidemics using extensive molecular analyses

The South American* C. gattii *AFLP6 population has the highest genetic diversity
AFLP fingerprinting, SCAR-MLST, microsatellite analysis and re-analysis of published MLST data (Fraser *et al.*, 2005; Byrnes *et al.*, 2010) was used to determinate the origins of *C. gattii* AFLP6 outbreaks. This revealed that South America (SA) has the highest genetic diversity and the largest effective population size (θ) compared to, and followed by, Australasia (AUS) and North America (NA) (Supplementary Table 4). This is in agreement with previous investigations that showed the presence of all *C. gattii* genotypes in South America, pointing towards an evolutionary origin on this continent (Meyer *et al.*, 2003; Ngamskulrungroj *et al.*, 2009; Trilles *et al.*, 2003). Alternatively, AUS has been proposed as the origin for the *C. gattii* AFLP6 outbreak at Vancouver Island as a result of same-sex between two less virulent lineages that gave rise to a hypervirulent lineage that was introduced in NA (Fraser *et al.*, 2005). However, the genetic background of the proposed non-virulent parental AFLP6B lineages was not found in the hypervirulent AFLP6A lineage. A third hypothesis is that *C. gattii* could be introduced in NA from any other locality (Kidd *et al.*, 2004). This latter hypothesis can be rejected since the current and other studies have shown that there is much more genetic diversity among globally collected *C. gattii* AFLP6 strains (Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Ngamskulrungroj *et al.*, 2009)

South America is the cradle of the global* C. gattii *AFLP6 population
Our data shows a SA origin for *C. gattii* AFLP6, more precisely in the Brazilian Amazone. The basal lineage is an environmental strain isolated from a native jungle tree in pristine Brazilian Amazon rainforest (Fortes *et al.*, 2002), indicating the presence of *C. gattii* AFLP6 in the environment of the Amazon and that introduction into SA is highly unlikely to have occurred via import of Australian *Eucalyptus* trees (Byrnes *et al.*, 2010; Fraser *et al.*, 2005). The hypothesis of a South American origin was originally thought to be unlikely, given the lack of an outbreak-related lineage (Fraser *et al.*, 2005). The presence of two SA strains that are indistinguishable from the Vancouver Island outbreak genotypes AFLP6A now makes this scenario more likely. A South American origin of the outbreak lineages is supported by a mitochondrial *COX2* locus that is identical among SA, Vancouver Island and Pacific Northwest lineages (Supplementary Figure

6 and 7). Given that the expression of mitochondrial encoded genes is up-regulated in hypervirulent *C. gattii* strains (Ma *et al.*, 2009) and mitochondrial recombination occurs in various *C. gattii* genotypes (Bovers *et al.*, 2009; Xu *et al.*, 2009) it is likely that intragenotypic mitochondrial recombination may contribute to the composition of hypervirulence traits.

A high virulent phenotype is present among ancient* C. gattii *AFLP6 lineages

The expanding Vancouver Island *C. gattii* AFLP6 outbreak has affected hundreds of humans and animals. The majority (≈90%) of the Vancouver Island outbreak strains belong to the highly virulent genotype AFLP6A that has only rarely been found outside of the outbreak region. Virtually all clinical cases have been caused by *C. gattii* AFLP6A, which is suggestive for a more hypervirulent phenotype than strains of genotype AFLP6B (Datta *et al.*, 2009; Fraser *et al.*, 2005; Kidd *et al.*, 2004). Relatively smaller outbreaks have been observed recently in Brazil and Australia, which were caused by *C. gattii* AFLP6B strains and thus show that the hypervirulent trait might not be restricted to the Vancouver Island outbreak-related genotype AFLP6A strains (Carriconde *et al.*, 2011; McGill *et al.*, 2009; Raso *et al.*, 2004). This is further strengthened by our observation that South American *C. gattii* AFLP6B strains occurring within the basal lineages of the coalescence-based gene genealogy were all found to be hypervirulent. Strain LA362 (H2) originated from a *C. gattii* AFLP6B outbreak among psittacine birds (Brazil) and showed a hypervirulent phenotype in both mice and macrophage host models (Figure 1 and 3). The minor genotype Vancouver Island outbreak genotype AFLP6B reference strain A1M-R272 (H3) was avirulent in the immunocompetent BALB/c mice model and lacked the ability to proliferate inside J774 macrophages (Ma *et al.*, 2009). Similar results for this strain were also observed by Fraser *et al.* (2005) and Ngamskulrungroj *et al.* (2010) using immunodeficient A/Jcr and immunocompetent BALB/c mice, respectively. The latter researchers found that eight genetically identical strains from AUS and NA possessed this hypovirulent phenotype, while two clinical strains from AUS within this lineage were highly pathogenic in the BALB/c mice model. Besides the fact that these different mice pathogenicity models revealed similar virulence outcomes for the tested *C. gattii* strains, it also indicates that a hypervirulent trait is more widely present among *C. gattii* AFLP6 strains than was assumed before.

Vancouver Island outbreak *C. gattii* AFLP6A strains were found to be highly virulent in two host models, irrespective of their clinical, veterinary or environmental source. However, the environmental strain CBS7750 isolated from a *Eucalyptus camaldulensis* tree in California has an avirulent phenotype in different host models (this study; Ma *et al.*, 2009; Ngamskulrungroj *et al.*, 2010). The clinical strain CBS6956 (= NIH444; H1) that is genetically indistinguishable from the Vancouver Island outbreak genotype AFLP6A/VGII was isolated from sputum of a patient in Seattle in the 1970s. This strain was repeatedly found to have an attenuated virulence (Fraser *et al.*, 2005; Ma *et al.*, 2009). Remarkably, this strain was isolated from a patient approximately 30 years prior the onset of the expanding Vancouver Island outbreak and was found to be genetically indistinguishable to the AFLP6A outbreak lineage. This indicates that the outbreak lineage was at least present for several decades in the environment of

the Pacific Northwest but that it did not cause an outbreak at that time. The South American strains IFM47258 and LA295 were genetically indistinguishable from the Vancouver Island outbreak lineage H1 and had a virulence phenotype similar to that of *C. gattii* AFLP6A Vancouver Island outbreak strains (Ngamskulrungroj *et al.*, 2010).

A high virulence potential in immunocompetent BALB/c mice and high proliferation rates in J774 macrophages occurs in strains occurring in basal lineages of *C. gattii* AFLP6, including the most basal lineage originating from a tree in the pristine Amazon rainforest. Thus the hypervirulent trait is not restricted to Vancouver Island outbreak strains, as was assumed previously (Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Ma *et al.*, 2009; Ngamskulrungroj *et al.*, 2010). Both hyper- and hypovirulent phenotypes were observed among and within recently emerged *C. gattii* AFLP6 lineages (Figure 1).

Cryptococcus gattii genotype AFLP6C strains from an outbreak that recently emerged in the Pacific Northwest had a similar virulence potential in mice and macrophage pathogenicity assays as strains from the outbreak locality in Canada (Byrnes *et al.*, 2010). These researchers suggested that the enhanced virulence potential of *C. gattii* AFLP6C strains might be partly due to recombination events with lineages from either Australia or South America (Byrnes *et al.*, 2010). The SCAR-MLST phylogenetic analysis (Figure 2) clearly shows that these genotype AFLP6C strains are not genetically closely related to the ongoing Vancouver Island outbreak lineages but that these strains cluster together with strains from South America. This points towards a genetic origin in South American of *C. gattii* AFLP6C and that this lineage emerged from recombination between South American strains as indicated by its phylogenetic and CASS recombination network position between other South American strains (Figure 2 and Supplementary Figure 3). Nevertheless, it seems that the AFLP6C lineage was introduced into North America independently from the Vancouver Island outbreak AFLP6A and AFLP6B lineages as suggested before (Byrnes *et al.*, 2009, 2010).

Range expansion of C. gattii *due to climate change and other factors*

During the past two decades several *C. gattii* AFLP6 outbreaks have been observed. Besides the ongoing outbreak of *C. gattii* in the Pacific Northwest, smaller outbreaks have been documented at other localities, including outbreaks among sheep in Western Australia since 1993, an outbreak among psittacine birds in Brazil where high numbers of *C. gattii* infections have been reported among immunocompetent native children living in the Amazon province of Pará, Brazil (Carriconde *et al.*, 2011; Raso *et al.*, 2004; Santos *et al.*, 2008, Trilles *et al.*, 2008). During the same period, a genotype of *C. gattii* known as AFLP4/VGI has emerged in the European Mediterranean area where it has caused several small outbreaks among goats, and a recent environmental sampling survey revealed that *C. gattii* AFLP4/VGI occurs widespread in autochthonous tree species from the Spanish Mediterranean region (Colom *et al.*, 2011). These examples show clearly that *C. gattii* is heading from the previously endemic tropical and subtropical regions, such as the Brazilian Amazone, towards the more temperate climate zones like the European Mediterranean region.

According to the recently updated Köppen-Geigen climate classification system (Peel *et al.*, 2007), the outbreak localities in Western Australia, Mediterranean Europe and in North America are all situated in regions with a Mediterranean sea climate, which is characterized by either dry summers or dry winters with an average temperature of 22°C during the warmest month. The results of the current study exclude the sudden emergence of a single hypervirulent lineage that may have given rise to the various outbreaks. Global warming may have an important role since there is a correlation between this and the timing of the recent outbreaks of *C. gattii* (Levitus *et al.*, 2001). Estimates of climate change velocity revealed that temperate and Mediterranean climate zones fell into the group that has the highest velocity in temperature change (Loarie *et al.*, 2009). An increase in the average annual temperature might influence the emergence and severity of pathogenic microorganisms in temperate climate zones. An example of this is the plant pathogenic fungus *Phytophthora cinnamomi*, the cause of Mediterranean oak decline. This fungus is able to cause more severe root rot at temperatures higher than that currently occur in the Mediterranean region (Harvell *et al.*, 2002). The ongoing global temperature increase and the factors that are directly related to this, such as precipitation, humidity, air and water temperature, has changed the distribution patterns of a variety of pathogenic microorganisms affecting plants and animals (Harvell *et al.*, 2002; Jones *et al.*, 2008).

Global dispersal of C. gattii *AFLP6*

The *C. gattii* AFLP6 coalescence model showed that the oldest lineages originated from Brazil. Approximately 22,000 years ago a lineage dispersed to Africa, followed by a massive dispersal and recombination event approximately 9,000 years ago that introduced *C. gattii* AFLP6 into Australia, Europe and North America. Many lineages descended from this global dispersal event. The most remarkable of these lineages (H3) was introduced into all inhabited continents and this has led to recent outbreaks in Australia and North America during the past two decades. It remains unclear what mechanism, if not multiple mechanisms, were the cause for this ancient global dispersal. It seems most likely that recombination events played an important role to shape the genetic armamentarium of *C. gattii* AFLP6 to become favorably adapted to ecological zones where the species has not been reported before (Byrnes *et al.*, 2010). Most of the lineages with a South American origin emerged during the past 6000 to 7000 years and were apparently formed due to a high rate of recombination events which seems to be the driving force behind the genetic diversification in the South American Amazone, compared to *C. gattii* AFLP6 populations from other regions in the world. The time scale for the genetic diversification must be regarded as an absolute minimum estimate and given values might be higher than observed. The time scale is depends largely on the generation time, which was estimated under laboratory circumstance using *myo*-inositol as the sole carbon source. Most likely, the generation time of *C. gattii* AFLP6 might be longer under natural conditions as has been observed for *C. neoformans* cultured in soil and tree debris (Botes *et al.*, 2009; Ishaq *et al.*, 1968; Kidd *et al.*, 2007b).

Long distance dispersal may have contributed to the intercontinental dispersal of *C. gattii*. Unusual extreme weather events, such as cyclonic winds, may have a great effect on the dispersal of microbial organisms. For example, the plant pathogen *Puccinia melanocephala* caused devastating crop losses after it spores were introduced in the Caribbean by cyclonic winds from Western Africa in 1978 (Brown & Hovmøller, 2002). Another example is the intercontinental spread by human migration and subsequent aerial dispersal of *Mycosphaerella fijiensis* which is causing worldwide huge damages to banana plantations (Brown & Hovmøller, 2002). Caribbean coral reefs have been under threat by *Aspergillus sydowii* after this fungus was introduced by large dust deposits that originated from dust storms in Sahara and Sahel (Griffin, 2007). Aerial movements of human pathogen fungi have not been investigated at a large scale, but it has been shown that fungi, including *Cryptococcus* species, are present in dust storm samples and this might be a possible dispersal mechanism for the global distribution of *C. gattii* (Griffin, 2007). Besides air as a possible source for the propagation of *C. gattii*, it might be that also sea- and freshwater plays an important role in the dispersion. It is known that *C. gattii* can survive in water with salinity comparable to that of sea water and it has recently been observed that *C. gattii* AFLP6 is present in sea water and that many sea animals were found to be infected with *C. gattii* AFLP6 along the coastline of Western North America (Duncan *et al.*, 2006; Kidd *et al.*, 2004, 2007b).

In conclusion, coalescence gene genealogy analysis showed that the recent emergence of the various *C. gattii* AFLP6 outbreaks into temperate Mediterranean climate regions was due to ancient dispersal out of the tropical Amazon rainforest. Also, it has been demonstrated that the hypervirulent trait is not solely restricted to the outbreak lineages AFLP6A and AFLP6C from Canada and the USA, but that hypervirulence is widely present among all globally sampled lineages including the basal lineage that originated from pristine Amazon rainforest. This indicates that recombination events, that have been demonstrated to occur in high rates within the South American population, might be the driving force behind the diversification and the ongoing presence of hypervirulence among some lineages. It seems likely that global climate change has provided a perfect ecological niche for *C. gattii* in temperate climate zones where ideal conditions may prevail for population expansion and massive dispersal after adaptation to the local environment that had led to increased inoculums sizes and higher clinical and veterinary infection rates.

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Supplementary data

Upon request, Supplementary data will be provided by the authors.

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Chapter 9

The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation

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Summary

In 1999, the population of Vancouver Island, Canada, began to experience an outbreak of a fatal fungal disease caused by a highly virulent lineage of *Cryptococcus gattii*. This organism has recently spread to the Canadian mainland and Pacific Northwest, but the molecular cause of the outbreak remains unknown. Here we show that the Vancouver Island Outbreak (VIO) strains have dramatically increased their ability to replicate within macrophages of the mammalian immune system in comparison with other *C. gattii* strains. We further demonstrate that such enhanced intracellular parasitism is directly linked to virulence in a murine model of cryptococcosis, suggesting that this phenotype may be the cause of the outbreak. Finally, microarray studies on 24 *C. gattii* strains reveals that the hypervirulence of the VIO isolates is characterized by the up-regulation of a large group of genes, many of which are encoded by mitochondrial genome or associated with mitochondrial activities. This expression profile correlates with an unusual mitochondrial morphology exhibited by the VIO strains after phagocytosis. Our data thus demonstrate for the first time that the intracellular parasitism of macrophages is a key driver of a human disease outbreak, a finding that has significant implications for a wide range of other human pathogens.

Introduction

For many pathogens intracellular survival is critical to their pathogenicity because it provides a basis for dissemination and latency. Intracellular pathogens are able to spread within the body, triggering little or no effective immune response from the host. Moreover, many pathogens use the host cell as a nutrient source to support rapid replication, thus acting as genuine intracellular parasites (Nguyen & Pieters, 2005; Pelchen-Matthews *et al.*, 2004). The human fungal pathogen *Cryptococcus* is an intracellular parasite of macrophages and is believed to exploit these host cells in order to traffic from the lung, the primary site of infection, into the central nervous system (Charlier *et al.*, 2009; Chretien *et al.*, 2002; Feldmesser *et al.*, 2000; Tucker & Casadevall, 2002).

There are two pathogenic species within the *Cryptococcus* genus: *C. neoformans* and *C. gattii*. They are the causative agents of cryptococcosis, a fatal infection of the central nervous system in humans. Globally, the vast majority of cryptococcosis cases occur in immunocompromised patients and are caused by *C. neoformans*. In contrast, *C. gattii* typically infects immunocompetent individuals but causes only 1% of cryptococcosis cases worldwide and has thus been significantly understudied (Hull & Heitman, 2002). However, the ongoing outbreak of *C. gattii* infection in immunocompetent individuals on Vancouver Island and the recent spread of this outbreak to mainland British Columbia and the Pacific Northwest have dramatically increased global concern about this pathogenic species (Byrnes *et al.*, 2009a, 2009b; Kidd *et al.*, 2004). Previous Amplified Fragment Length Polymorphism (AFLP) and Multi-Locus Sequence Typing (MLST) studies have demonstrated that the Vancouver Island Outbreak (VIO) is mainly caused by a single, hypervirulent genotype of *C. gattii* (AFLP6A/VGIIa) (Kidd *et al.*, 2004). Intriguingly, this genotype is not restricted to Vancouver Island but is also shared by, for instance, the CBS6956 strain (also known as NIH444 or ATCC32609, isolated from a patient in Seattle in 1971) and CBS7750 (isolated from a *Eucalyptus* tree in San Francisco in 1992), suggesting that a recent genetic change has occurred within the VIO lineage, potentially as a result of an unusual same-sex mating event (Fraser *et al.*, 2005; Kidd *et al.*, 2005). However, the underlying molecular cause for the hypervirulence of this lineage remains unknown.

Materials and Methods

Yeast strains and growth conditions

All cryptococcal strains used are listed in Supplementary Table I. Yeast cells were grown overnight in antibiotic-containing YPD medium (2% glucose, 1% peptone and 1% yeast extract) with moderate shaking (240 rpm) at 25°C. Antibiotics were added to the media at the following concentrations: ampicillin 50 μg/ml or kanamycin 25 μg/ml. Yeast cells were collected from the overnight culture by centrifugation at 2000 rpm for 2 minutes, then washed with PBS twice and resuspended in PBS.

Intracellular proliferation measurement

A proliferation assay was developed to monitor intracellular proliferation rate of individual strains for a 64 hour period following phagocytosis. For this assay, J774 macrophage cells or human primary blood-derived macrophage cells (HPBMC, which were isolated and activated as previously described (Ma *et al.*, 2006)) were exposed to cryptococci opsonised with 18B7 antibody for 2 hours as described in (Ma *et al.*, 2006). Each well was washed with PBS four to five times to remove as many extracellular yeast cells as possible and 1 ml of fresh serum-free DMEM was added. For time point T_0, the 1 ml DMEM was discarded and 200 µl of sterile dH_2O was added into wells to lyse macrophage cells. After 30 minutes, the intracellular yeast were released and collected. Another 200 µl dH_2O was added to each well to collect the remaining yeast cells. The intracellular yeast was then mixed with Trypan Blue at a 1:1 ratio and the live yeast cells were counted. For the subsequent five time points T_{16}, T_{24}, T_{40}, T_{48} and T_{64}, both extracellular and intracellular cryptococci were collected and independently counted by haemocytometer in the same manner. For each strain tested, each time course was repeated at least three times on different occasions and using different batches of macrophages.

Intracellular proliferation rate was calculated by dividing the maximum intracellular yeast number (which is T_{24} for most of the strains) by the initial intracellular yeast number at T_0. We confirmed that Trypan Blue stains 100% of yeast in a heat-killed culture, but only approximately 5% of a standard overnight culture. Compared to conventional colony counting method, this method is more sensitive in detecting the clustered yeast population or yeast cells undergoing budding.

Mitochondrial staining and microscopy

Yeast cells, grown overnight in YPD medium at 25°C at 240 rpm, or grown at 37°C in DMEM in a 5% CO_2 incubator without shaking for 24h, or isolated from macrophages 24 hours after infection, were harvested, washed with PBS twice and resuspended in PBS containing the MitoTracker Red CMXRos (Invitrogen, Paisley, United Kingdom) at a final concentration of 40 nM. Cells were incubated for 30 min at 37°C. After staining, cells were washed three times and resuspended in PBS. For each condition, more than 60 yeast cells for each of the tested strains were chosen randomly and analyzed. For quantifying different mitochondrial morphologies, images were collected on a Nikon Eclipse E300 microscope using 60× oil immersion 1.40NA plan APO objective lens. Both fluorescence images and bright field images were collected simultaneously. Images were captured with identical settings on a Hammatsu Orca C4745-12NRB with a 0.5× camera lens using Openlab v5.5.0 (Improvision, Coventry, United Kingdom). All Images were processed identically in Photoshop CS2 (Adobe, San José, CA) and mitochondrial morphologies were analyzed and counted blind. For confocal microscopy, images were collected on a Nikon Eclipse E600 confocal microscope with BioRad Radiance 2000 MP laser scanning system using a 100× oil immersion WD 0.20 objective lens. Images were processed using Zeiss Sharp2000 software v6.0 (Carl Zeiss, Rugby, United Kingdom).

Mouse survival assay

Yeast inocula (10^6 yeast cells per mouse from a 48 hours culture of cryptococci on Sabouraud dextrose agar (SDA) at 30°C) were injected into the tail veins of male albino BALB/c mice (weight, 20-23 g) by administration of a single bolus injection of a 0.2 ml suspension in PBS. The yeast concentration was determined by counting with a haemocytometer. The viable count was measured as the number of CFU on SDA plates after 24 to 48 hours of incubation at 30°C. With these inocula, systemic infections are regularly produced in mice, and they cause total killing within 5 to 50 days. For each experiment 10 mice were used, maintained in separate cages. The number of surviving animals in each group was recorded daily over a period of 45 days using a blinded experimental design such that animal handlers were unaware of the corresponding IPR value for the strains used. All procedures, care and treatment of mice were in accordance with the principles of humane treatment outlined by the Guide for the Care and Use of Laboratory Animals of the Hebrew University, and were approved by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals (approval number OPRR-A01-5011).

Microarray – RNA isolation of intracellular cryptococci 24 hours post infection

The phagocytosis assay was carried out as described earlier. After 24 hours, extracellular cryptococci were removed with several pre-warmed PBS washes and macrophages were lysed with 10 ml ice-cold H_2O for 20 minutes before being scraped from T75 tissue flasks. The whole mixture was then centrifuged at 1500 rpm at 4°C for 5 minutes and the resulting pellet was washed twice with ice-cold H_2O. Subsequently, the pellet was resuspended in 10 ml of H_2O supplemented with 0.05% sodium dodecyl sulphate (SDS) for 5 minutes to remove macrophage RNA. At this SDS concentration, the macrophages were lysed while cryptococci remained intact and viable (Fan *et al.*, 2005). The remaining yeast cells were washed three times with ice-cold H_2O by centrifugation and resuspension. The final pellet was then used for RNA isolation or frozen at - 80°C until required. Yeast RNA was isolated using Micro-to-Midi Total RNA Purification System (Invitrogen, Paisley, United Kingdom) in accordance with the manufacturer's instructions.

Microarray – microarray experiment

An Agilent printed whole genome tiling array with 242,003 probes generated against two genomes of *C. gattii*, A1M-R265 (Broad Institute) and WM276 (Jim Kronstad, personal comm.), was designed by Oxford Gene Technology (OGT). Average interprobe distance was 140-nucleotides, evenly distributed across both DNA strands. After round one of design, any gaps of over 1.5 kb (due to repeat regions, etc) were then filled using suboptimal probes (700 probes in total) in the same manner. There are 20,212 probes (8.4%) that map onto both genomes.

Using this design, 24 *C. gattii* RNA samples were used to determine differences in their gene expression 24 hours after internalisation by macrophages. For the array experiment, RNA samples from each strain were labelled with Cy3, whilst the control, consisting of a pooled sample containing equal quantities of RNA samples from all 24 strains (reference) was labelled with Cy5. See Supplementary data for more experimental details.

Microarray – data analysis
Data were background subtracted using Bayesian method, normalized using Loess normalization (Stekel, 2003) and analyzed using the statistical package R (http://www.r-project.org/) based on linear regression against IPR values (an example script is included in the Supplementary Materials and methods). Data were corrected for false-discovery rate (Benjamini & Hochberg, 1995) before candidates showing q-values smaller than 0.086 were BLASTed against both *C. neoformans* and *C. gattii* databases. All hits were also retained for statistical analysis of probe distribution.

Real-time PCR
Real time PCR using the Sybr Green method was performed on the genomic DNA generated from newly isolated intracellular cryptococci to check the copy number of the mitochondrial genome in comparison to a nuclear region. For the reaction, four strains (ENV152, A1M-R271, CBS7750 and CBS8684) were chosen. Every reaction in the experiment was conducted in triplicate. For amplification of the nuclear locus the forward primer 5'-GGTCGAATTGTTCTCAGG-3' and the reversed primer 5'-ACCGTCCTTAACCTCGAC-3' were used, while for the mitochondrial locus the forward primer 5'-TTCGTCTTGCTGGTCGACTT-3' and reversed primer 5'-TGGGAGTTGTTGATCGTTG-3' were used.

Phenotypic analysis
Proteinase and phospholipase activities were measured as described by Braga and colleagues (1998) and Vidotto and colleagues (1996), respectively. Melanin production and capsule size were measured as described by Rosas and Casadevall (1997) and Golubev and Manukian (1979), respectively. Other enzymatic activities of *C. gattii* strains were tested with API-ZYM as described previously by Chen and co-workers (1997). Unless otherwise stated, statistical tests were performed by linear regression to test whether any of the tested phenotype was correlated with the intracellular proliferation rate.

Results

Hypervirulence of VIO strains is not directly linked to known virulence factors
To study the cause of hypervirulence within the VIO isolates, we undertook a high-throughput analysis of well-characterized virulence traits (capsule size, melanisation, phospholipase production, proteinase production and other enzymatic activities) in 39 *C. gattii* strains representing both VIO and non-VIO isolates (Supplementary Tables 1 and 2). However, there was no consistent difference between VIO and non-VIO isolates belonging to this genotype in any of the traits tested, suggesting that the virulence of VIO strains does not simply result from over-expression of these individual cryptococcal pathogenicity factors. Such a finding is consistent with recent data suggesting that many cryptococcal virulence genes/factors remain to be discovered (Liu *et al.*, 2008).

VIO strains show enhanced intracellular parasitic capacity

Given the importance of macrophage parasitism in cryptococcal infection, we developed an *in vitro* method to monitor intracellular yeast number for 64 hours following phagocytosis by murine macrophage-like cells J774 (see experimental procedures). We then used Intracellular Proliferation Rate (IPR, which was calculated by dividing the maximum intracellular yeast number by the initial intracellular yeast number at T_0) as a parameter to quantify the relative level of intracellular parasitism between strains. Based on this method, we calculated average IPR for 55 strains, including 16 *C. neoformans* and 39 *C. gattii* isolates (Supplementary Table 1). Strikingly, all of the VIO AFLP6A isolates exhibit much higher intracellular proliferation rates compared to other *C. gattii* strains, including AFLP6A strains isolated from other areas of the world (Figure 1A). Timelapse microscopy of J774 macrophages infected with each of the *C. gattii* strains confirmed the high IPR values observed among VIO strains (Supplementary Movies 1-3). Importantly, this phenomenon is not specific to the J774 mouse cell line, because the VIO strains also show enhanced intracellular proliferation in human primary macrophages derived from peripheral blood (Figure 1B and Supplementary Table 4). This proliferative capacity appears to reflect specific parasitism of the live macrophage, rather than simply enhanced utilisation of nutrient sources available in the host cell, since the VIO strains do not show significantly higher growth rates when grown in mammalian cell lysates (Supplementary Table 5). Thus the VIO strains share a common capacity to act as intracellular parasites that is unique to this pathogen population.

IPR predicts virulence of cryptococcal strains in a murine cryptococcosis model

Several early studies have highlighted the hypothesis that macrophages may exacerbate cryptococcal infection in mice. Alveolar macrophage depletion was associated with amelioration of disease in three murine strains as measured by lung fungal burden, and decreased the dissemination of a glucosylceramide-deficient mutant of *C. neoformans* in immunodeficient mice (Kechichian *et al.*, 2007; Shao *et al.*, 2005). We therefore tested whether high intracellular proliferation capacity is linked to the hypervirulence of the VIO lineage. To do this, IPR values were compared with both published and newly-generated mouse median survival times (ST_{50}). Remarkably, IPR and murine ST_{50} are highly significantly correlated (P=0.00017, linear regression, Figure 2), a correlation that holds true for both *C. neoformans* and *C. gattii* and is independent of the mouse model used. In other words, the ability to survive and proliferate inside a host macrophage contributes significantly to cryptococcal virulence in the murine model and presumably also in infected humans, as suggested by the human primary macrophage data. This previously undiscovered link between intracellular proliferation rate and virulence also provides a possible explanation for why "ancestral" AFLP6A strains share the VIO genotype and yet do not lead to disease outbreaks. For example, CBS6956, which is considered as the potential origin of the AFLP6A subtype (Fraser *et al.*, 2005; Kidd *et al.*, 2005), shows only a moderate IPR value of 1.35 (Figure 1A, Supplementary Table 1), indicating that it cannot exploit the intracellular macrophage niche as successfully as the VIO isolates.

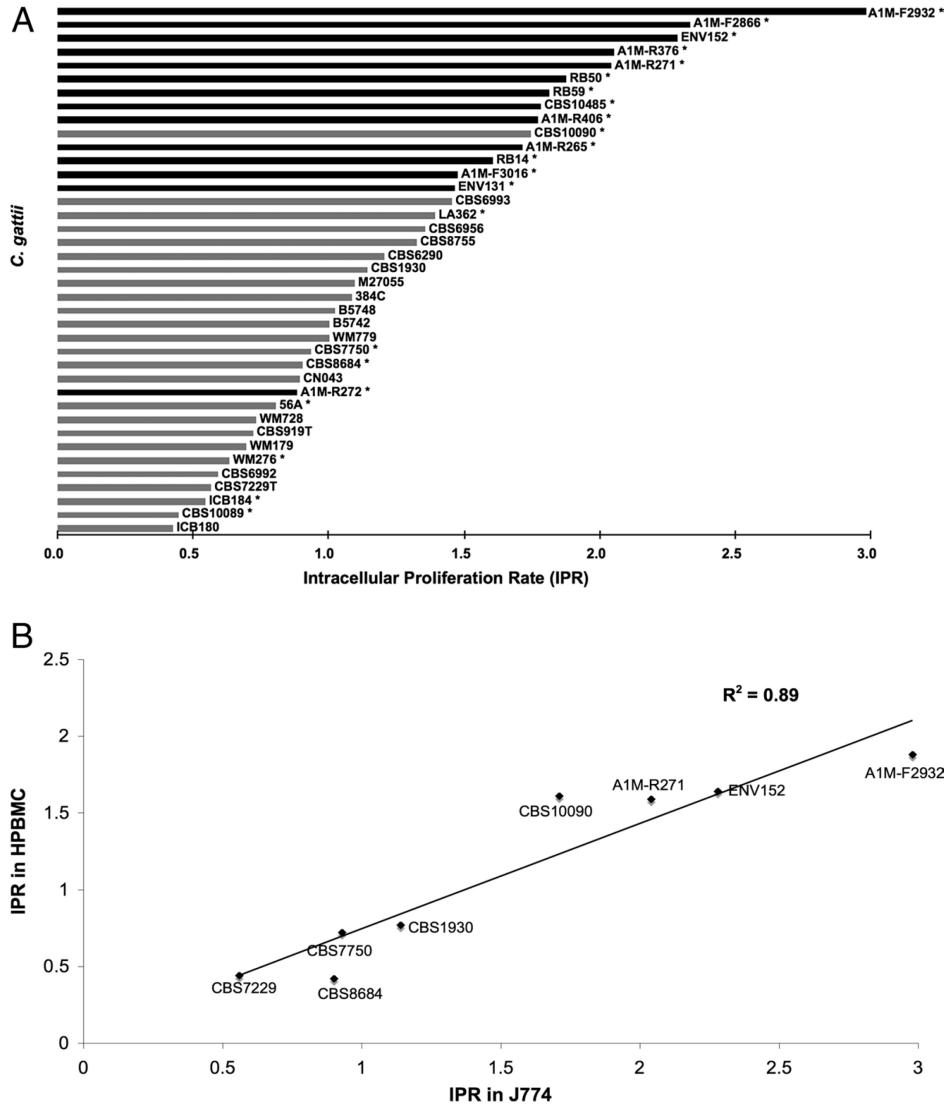


Figure 1. Panel A shows significant interstrain variation in IPR occurs within the *C. gattii* species (*n*=39). Black bars represent VIO isolates, which proliferate far better than other *C. gattii* strains (grey bars). The only exception was A1M-R272, which belongs to the minor (AFLP6B) group of the outbreak and has previously been shown to be less virulent than other VIO strains (Fraser *et al.*, 2005). An asterisk (*) denotes strains used for the microarray study. Panel B shows IPR values of eight strains in human primary blood-derived macrophage cells (HPBMC) correlate significantly with those observed in J774 (*P*=0.00044, Spearman's test, *n*=8). The IPR values obtained with HPBMC are generally lower than those seen in J774 macrophages, which may be a result of the higher cryptococcal expulsion rates observed in primary cells, as previously reported (Ma *et al.*, 2006).

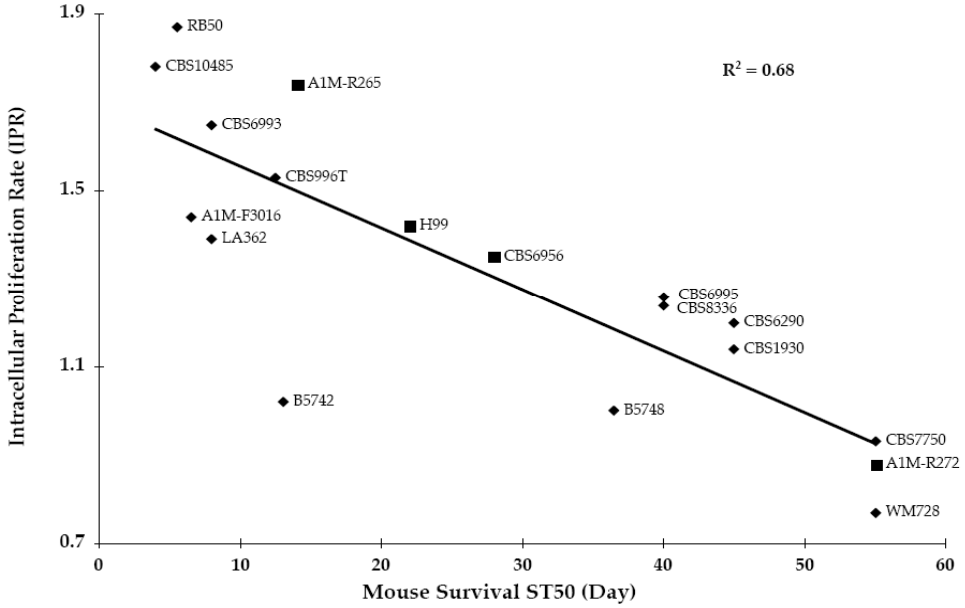


Figure 2. A significant correlation between mouse survival data (both previously published (Fraser *et al.*, 2005) [■] and newly-generated [◆]) and intracellular proliferation rate (IPR). Mice survived longer when infected with strains with low IPR values as compared with animals infected with high IPR strains (P=0.00017, linear regression, n=18). For the unpublished mouse survival assays, experiments were conducted as described in the Materials and Methods section. Published mouse survival data are taken from Fraser and colleagues (2005). Note that strain WM276 has been omitted from the graph, since published ST_{50} values on this strain vary widely for reasons that are not currently understood (Chaturvedi et al., 2005; Fraser *et al.*, 2005). For strains that did not cause mortality of 50% or more within the 45-day timeframe of the experiment, we arbitrarily assigned an ST_{50} of 55 days.

Identification of genes associated with enhanced intracellular parasitism

Statistical analysis confirmed that the high IPR values exhibited by the VIO isolates did not result from increased expression of any of the known virulence factors analyzed above (Supplementary Table 2B) nor was it due to better utilisation of macrophage nutrients (Supplementary Table 5). Therefore in order to investigate the molecular basis of enhanced intracellular proliferation, and thus hypervirulence, in the VIO isolates, we used custom-designed *C. gattii* whole-genome tiling microarrays to conduct transcriptional profiling of 24 *C. gattii* strains (21 AFLP6 and 3 AFLP4 isolates) recovered from within J774 macrophages. These strains are genetically very similar but show a wide range of IPR values (Figure 1A). RNA samples from each strain were isolated from intracellular cryptococcal cells 24 hours after infection and competitively hybridized against a pooled sample containing equal quantities of RNA from all 24 strains. At this timepoint, the intracellular number reaches the peak for the majority of strains. Linear regression identified 1367 target loci in the genome of AFLP6 strains

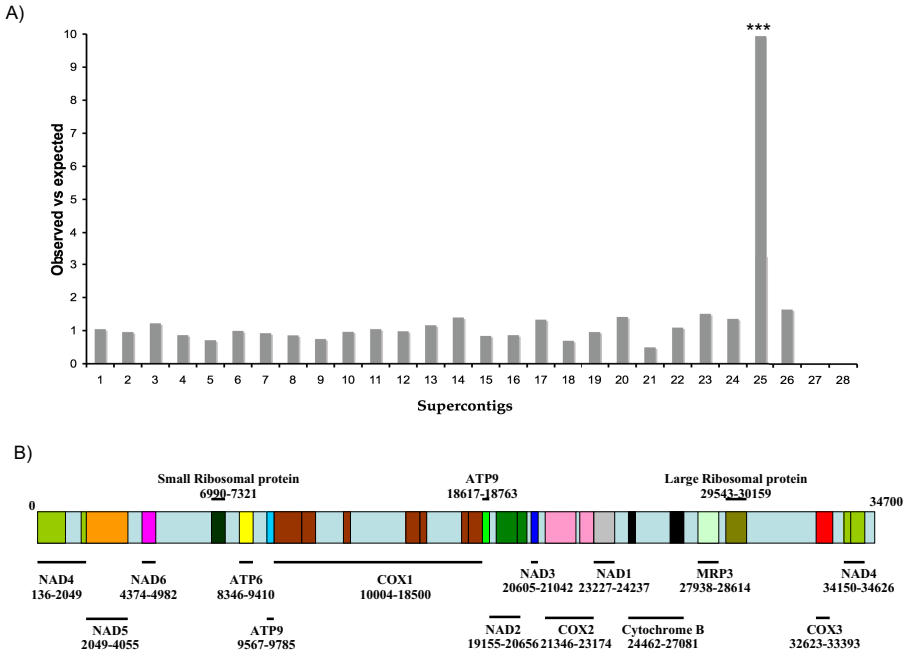


Figure 3. Panel A shows that A1M-R265 Supercontig 25 is highly over-represented in the transcriptional analysis (P=10^{-24}, Chi-square test). 1367 probes whose expression showed significant correlation with IPR were mapped onto the 28 supercontigs of the A1M-R265 genome and compared to the expected hit rate (assuming a random probe distribution) using the Chi-square test. Supercontig 25 is ten-fold over-represented. Panel B shows the predicted genome structure of A1M-R265 supercontig 25. The genome is 34.7 kb in size and, as with other cryptococcal mitochondria, encodes 17 genes important for mitochondrial function and protein synthesis. The open reading frames were predicted using Open Reading Frame Finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) in combination with alignment to the H99 mitochondrial sequence (Genbank accession number AY101381).The supercontig represents the whole mitochondrial genome of A1M-R265. Sections with light blue colour are either introns or intergenic spaces, both of which are substantially expanded relative to *C. neoformans* var. *grubii* strain H99.

whose expression showed a significant correlation with IPR values. 224 of these have predicted function annotations, most of which can be categorized into one of five groups: carbohydrate metabolism, stress response, vesicle/vacuole fusion and transport, protein degradation and synthesis, and nucleotide metabolism (Supplementary Table 3). Interestingly, although our phenotypic analysis demonstrated that no single known virulence factor was responsible for the hypervirulence of the VIO isolates, several of these genes (e.g. *CRG1*, *PLB1*, capsule related genes and genes on the mating-type locus) showed a significant expression correlation with IPR, suggesting that they may synergistically contribute to virulence within the AFLP6 lineage. Notably, some of our uncharacterized candidate genes were also recently identified by Liu and colleagues

(2008) as influencing melanin and capsule production and thus cryptococcal infectivity (e.g. *COP9* signalosome complex, ubiquitin carboxyl-terminal hydrolase) (Liu *et al.*, 2008).

Due to poor annotation of the *C. gattii* genome, BLAST searching did not identify the function of most of the candidate genes we identified (Supplementary Table 3). We therefore mapped the genomic distribution of all the microarray hits by localising them on the 28 supercontigs of A1M-R265 genome. The distribution was homogenous across all supercontigs, with the remarkable exception of supercontig 25, corresponding to the mitochondrial genome (mtDNA), which was ten-fold over-represented (Figure 3A; $P=10^{-24}$, Chi-square test). Quantitative PCR demonstrated that the observed over-representation of mitochondrial sequences is not due to an increase in mtDNA copy number, because AFLP6 strains showing very different IPR values nonetheless have similar mtDNA/genomic DNA ratios (Table 1). In addition, mtDNA copy number does not change significantly following intracellular replication, eliminating this as a possible explanation for the overrepresentation of mitochondrial hits (Table 1). Furthermore, the expression of many nuclear-encoded proteins that function in mitochondria is also up-regulated in the VIO strains (e.g. respiratory genes and mitochondrial proteins, Supplementary Table 3), which is particularly relevant given early *in vivo* transcriptional profiling showing the high expression of several respiratory genes by this yeast at the site of a central nervous system infection (Steen *et al.*, 2003; Toffaletti *et al.*, 2003). Taken together, we propose that mitochondrial function is critical for the virulence of this lineage.

Table 1. Real-time PCR to quantify mtDNA copy number per cell.

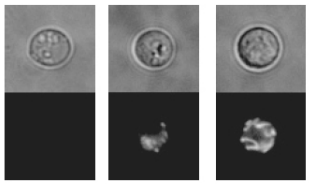
	Real-time cycle difference between mitochondrial and nuclear loci	
Strain	**Intracellular yeast cells**	**Yeast cells grown in YPD at 25°C**
ENV152	9.75 ± 1.01	9.77 ± 0.61
A1M-R271	8.74 ± 0.38	10.68 ± 1.04
CBS7750	9.44 ± 0.19	10.14 ± 1.59
CBS8684	10.71 ± 0.63	10.44 ± 0.53

Cryptococcal mtDNA copy number (ranging from 400 to 1,600 copies per cell) does not vary among different AFLP6 strains (two VIO isolates and two other AFLP6 strains) or change following intracellular replication within host macrophages.

Mitochondrial morphology changes in VIO strains following phagocytosis

Given the over-representation of mitochondrial genes in the VIO strains, we analyzed the morphology of cryptococcal mitochondria both before and after phagocytosis. Surprisingly, we observed a striking difference in mitochondrial morphology between the VIO and non-VIO strains following intracellular parasitism. During *in vitro* growth either at 25°C with shaking in YPD or 37°C in DMEM + 5% CO_2 without shaking, more

A



Strains	Diffuse	Globular	Tubular	Total	Percentage of tubular	IPR values
CBS8684	15	46	0	61	0%	0.90
CBS7750	9	58	4	71	6%	0.93
CBS1930	10	44	6	60	10%	1.14
A1M-R271	7	32	24	63	38%	2.04
ENV152	11	28	39	78	50%	2.25
A1M-F2932	14	21	54	89	61%	2.98

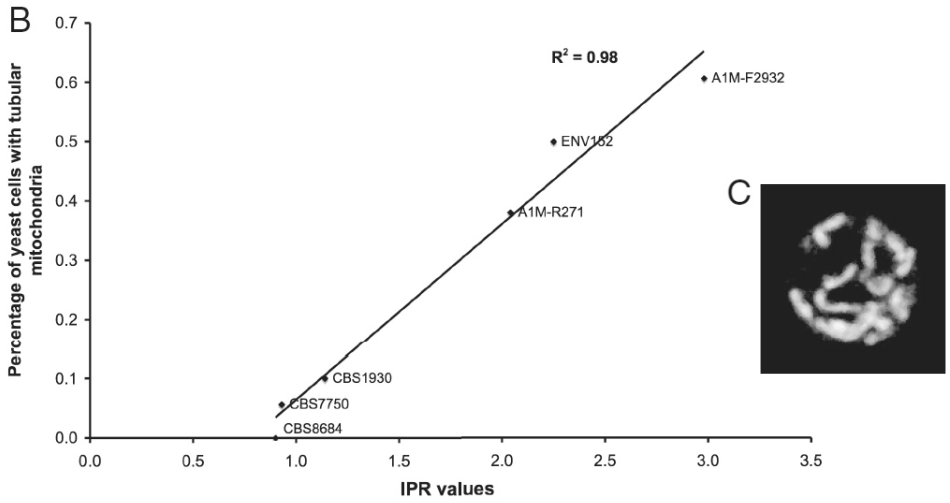


Figure 4. Panel A shows representative images of the three different mitochondrial morphologies observed (diffuse, globular and tubular) and a table showing the percentage of intracellular yeast cells with tubular mitochondria in six AFLP6 strains (three VIO and three non-VIO isolates). For each strain, a random selection of cryptococcal cells were scored blindly for the three different mitochondrial morphologies. Mitochondria with a tubular morphology were found only rarely in strains with low IPR values (non-VIO strains) or in strains (both VIO and non-VIO) that had been grown extracellularly. Panel B present the percentage of intracellular yeast exhibiting a tubular mitochondrial morphology correlates significantly with IPR value (n=6; P=0.00021, linear regression). In Panel C, a Z-projection confocal image showing the tubular mitochondrial morphology of a VIO strain. *Cryptococcus gattii* strain ENV152 (IPR=2.28), isolated after 24 hours of growth within J774 macrophages and labelled with MitoTracker.

than 95% of cryptococcal cells have mitochondria with morphologies that we termed either "diffuse" or "globular" (Figure 4A), regardless of the strain tested. However, after growth inside J774 macrophages, VIO strains developed a tubular mitochondrial morphology (Figure 4C) that was rarely exhibited by non-VIO isolates ($P<0.0001$, Chi-square test for VIO versus non-VIO strains). Remarkably, the percentage of cryptococcal cells exhibiting tubular mitochondria shows a strong, linear correlation with IPR (Figure 4B; $P=0.00021$, linear regression), a relationship that raises the possibility of accurately predicting the virulence of novel cryptococcal genotypes based on a simple, one-step observation of mitochondrial morphology.

Mitochondrial tubular morphology is generally thought to result from mitochondrial fusion, a phenomenon that allows mitochondria within a cell to cooperate with each other and protects cells from the detrimental effect of mtDNA mutations by allowing functional complementation of mtDNA gene products (Chan, 2006; Chen *et al.*, 2003). Moreover, mitochondrial fusion has been found to protect cells from cell death (Karbowski *et al.*, 2004; Sugioka *et al.*, 2004). It therefore appears likely that the altered mitochondrial gene expression and morphology seen in the VIO strains is a protective response that facilitates rapid intracellular growth and thus enhanced virulence.

Discussion

Our study demonstrates a link between the intracellular parasitism of phagocytes and the virulence of a facultative intracellular pathogen in a murine model of infection. Moreover, we propose that a recent change in mitochondrial regulation within the *C. gattii* lineage has led to an increased intracellular proliferative capacity resulting in the hypervirulent phenotype that underlies the VIO. This change leads to a quantitative linear relationship between intracellular proliferation rate, mitochondrial gene expression, mitochondrial morphology and virulence.

The mitochondrion, as an essential organelle, has been linked to various cellular activities, such as intermediary metabolism and respiration, cell signalling, iron metabolism, apoptosis and aging. Nevertheless, its role in modulating virulence of pathogens is unclear. Indeed such a role has been reported only once before, in *Heterobasidion annosum*, which, like *Cryptococcus*, is also a basidiomycete pathogen but of plants rather than animals (Olson & Stenlid, 2001). However, mitochondria can represent a source of rapid evolution of virulence in emerging pathogens because their genomes are present at high copy number and show a mutation rate much higher than that of nuclear DNA (Denver *et al.*, 2000; Haag-Liautard *et al.*, 2008; Lynch *et al.*, 2008). Within the *Cryptococcus* genus, mitochondrial genomes show conserved gene synteny but very different sizes (e.g. 34.7 kb for *C. gattii* (Figure 3B), 32 kb for *C. neoformans* var. *neoformans*, and 24 kb for *C. neoformans* var. *grubii*; Litter *et al.*, 2005), indicating that they are under intense selection. This is further supported by recent evidence of mitochondrial recombination in *C. gattii* (Bovers *et al.*, 2009; Xu *et al.*, 2009), along with the fact that most of the mitochondrial genetic variation between WM276 (an

AFLP4/VGI strain with a low IPR value) and A1M-R265 (a VIO strain with high IPR) lies in coding regions, whilst the intergenic regions are highly conserved.

Mitochondria are dynamic organelles that frequently divide and fuse with each other and it is thought that such behaviours are coordinated with their metabolic function (Chan, 2006). In yeast and mammals, several factors including *Drp1*/*Dnm1* and *Mfn*/*Fzo1* are known to regulate mitochondrial morphology by controlling membrane fission or fusion. Interestingly, we find that *FZO1* is up-regulated in the VIO strains (Supplementary Table 3) and might therefore contribute to the observed tubular morphology in VIO strains. However, a recent study demonstrated that most fundamental mitochondrial functions, including metabolism and oxidative phosphorylation, are also necessary for the maintenance of tubular networks (Ichishita *et al.*, 2008). Hence the tubular morphology is likely to be a result of upregulation of many candidate genes listed in Supplementary Table 3. Early studies on *Cryptococcus* have demonstrated the importance of mitochondria in responding to hypoxic conditions and oxidative stress (Ingavale *et al.*, 2008; Narasipura *et al.*, 2005). We therefore propose that, after being engulfed by macrophages, the VIO strains are able to promote mitochondrial fusion to form long tubular mitochondria in order to more efficiently repair mtDNA damage caused by the oxidative species and hypoxic conditions present within the macrophage phagosome.

Since mitochondria are essential for cryptococcal viability (Toffaletti *et al.*, 2004), attempts to make petite (respiratory) mutants have not been successful. In addition, we and others have been unable to mate AFLP6 mating-type **a** with mating-type α cryptococci in order to replace the mitochondria of VIO strains with those from less virulent strains (Fraser *et al.*, 2003; Halliday & Carter, 2003; Ngamskulrungroj *et al.*, 2008). Therefore, we cannot distinguish whether it is the mitochondrion itself or the regulation of mitochondrial activity via nuclear encoded proteins that is important for the virulence of the VIO lineage. In either scenario, however, mitochondrial regulated intracellular replication capacity may be a widespread phenomenon in other eukaryotic pathogens and hence an improved appreciation of this process is likely to have significant implications for our understanding of disease epidemics caused by a range of otherwise unrelated pathogens.

Acknowledgements

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Supplementary data

Supplementary data is only available at http://www.pnas.org/content/106/31/12980/suppl/DCSupplemental.

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Chapter 10

Cryptococcus gattii: risk for tourists visiting Vancouver Island, Canada

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To the Editor: An unprecedented outbreak by *Cryptococcus gattii* genotype AFLP6/VGII at Vancouver Island is affecting both human and animal hosts with normal immunity (Hoang *et al.*, 2004; Kidd *et al.*, 2004; Stephen *et al.*, 2002). Until now, more than hundred human cases, including at least six fatalities, have been reported by the British Columbia Centre for Disease Control (MacDougall *et al.*, 2006; http://www.bccdc.org/). Vancouver Island is a major tourist destination with annually approximately 7.5 million tourist visits (http://www.bcstats.gov.bc.ca/). Here, we report the first intercontinental transmission of *C. gattii* from this outbreak by a Danish tourist six weeks after returning from a holiday at Vancouver Island, indicating a potential risk for tourism-related acquirement of cryptococcosis.

A 51-year-old HIV-negative, apparently immunocompetent man, with known psoriatic gout on treatment with a non-steroid anti-inflammatory drug, was admitted due to chest pain, radiating to the left shoulder and arm, lasting for one day. Six weeks before admission he returned from a three-week holiday in Canada, where he visited Victoria and surroundings on the Eastern coast of Vancouver Island for seven days. During their stay the patient and his three fellow travellers visited gardens and the local natural vegetation.

During his stay in Canada the patient had no symptoms, and none of the family members developed any symptoms of cryptococcosis so far (September 2006). On admission at the Herning hospital (Herning, Denmark) his temperature was 38.2°C and a chest radiograph showed three large nodular infiltrates suspect for malignancy or abscesses. Neither bacterial nor fungal pathogens could be isolated from sputum by classical and molecular methods. After 4-5 days his temperature was 40°C, a productive cough with dyspnoea was noted and his condition deteriorated. A chest radiograph showed progression of the infiltrates and a CT scan of the abdomen and chest showed infiltrates near the pleura suggesting encapsulated fluid (Figure 1).

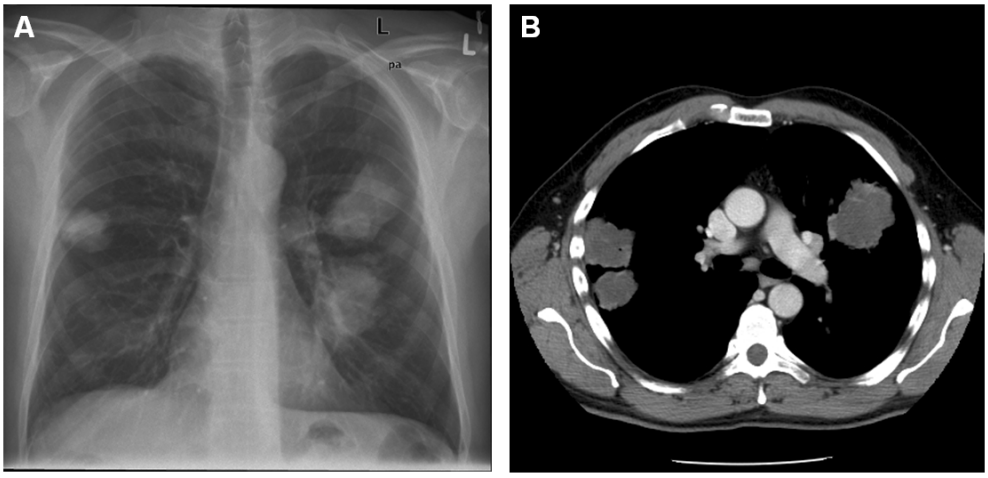

Figure 1. Panel A shows a chest radiograph and panel B a X-ray computed tomography scan showing three nodular *Cryptococcus gattii* infiltrates near the pleura.

An ultrasound-guided lung biopsy was performed and mucoid material was aspirated. Microscopy and a culture from the aspirate revealed a cryptococcal isolate. This isolate was further identified by ITS and D1/D2 sequencing, as well as AFLP analysis (Kidd *et al.*, 2004). In addition, detailed genotyping was performed using sequences of seven genes (*CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1* and *TEF1*α; GenBank accession numbers: DQ861593-DQ861599) (Fraser *et al.*, 2005).

Extensive molecular research revealed that this isolate belonged to the highly virulent genotype AFLP6A (= fingerprint type VGIIa) of *Cryptococcus gattii*, which is the major genotype involved in the Vancouver Island *C. gattii* outbreak (VIO) (Hoang *et al.*, 2004; Kidd *et al.*, 2004; MacDougall *et al.*, 2006; Stephen *et al.*, 2002). All seven sequenced genes had a complete match with the sequence-types specific for isolates involved in the VIO (Fraser *et al.*, 2005). Thus we conclude that the disease was attracted during a holiday at Vancouver Island and imported to Denmark. The presence of three cryptococcal masses of more or less equal size suggests that the patient was exposed to a high concentration of infectious cells of *C. gattii*. The observed incubation time of six weeks is shorter than that previously reported for VIO-related infections, which ranged between two and eleven months (MacDougall *et al.*, 2006). These observations, in combination with the absence of any known predisposing factor in this patient (such as smoking or treatment with corticosteroids) suggest that this specific AFLP6 genotype of *C. gattii* is highly virulent (Fraser *et al.*, 2005; MacDougall *et al.*, 2006).

The case here presented suggests a potential risk for tourists acquiring cryptococcosis while staying on Vancouver Island. Therefore, we recommend tourists and medical staff of health care centres worldwide to be alert of symptoms of cryptococcosis after travelling to Vancouver Island, British Columbia, Canada.

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Chapter 11

Activated dormant *Cryptococcus gattii* infection in a Dutch tourist who visited Vancouver Island (Canada): a molecular epidemiological approach

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Summary

An ongoing outbreak of *Cryptococcus gattii,* which emerged on Vancouver Island and the Pacific Northwest, has caused infections in more than 200 of the islands' residents, of whom eight have died. In travelers, *C. gattii* infections are rarely described. Here, we report a case of cryptococcosis in a patient treated with high dose corticosteroids for systemic lupus erythematosus induced autoimmune hemolytic anemia. She acquired *C. gattii* during a visit to Vancouver Island one year before the onset of the symptoms. This indicates that *C. gattii* may be present as a dormant infection that can be activated during treatment with corticosteroids.

Introduction

The primary pathogenic basidiomycetous yeast *Cryptococcus gattii* causes a life-threatening disease of the central nervous system, lungs and skin in humans and animals (Bovers *et al.*, 2008). *Cryptococcus gattii* occurs in tropical and sub-tropical regions of South America, Africa, Asia and Australia (Fraser *et al.*, 2005; Kidd *et al.*, 2004). Since 1999, an ongoing outbreak of *C. gattii* AFLP6/VGII emerged in the temperate climate of Vancouver Island (British Columbia, Canada). This outbreak has affected more than 200 humans and many hundreds of animals (Galanis *et al.*, 2007; Hoang *et al.*, 2002; Kidd *et al.*, 2004; Stephen *et al.*, 2002). Recently, it was shown that *C. gattii* has also colonized the mainland of British Columbia (Canada) and the Pacific Northwest of the United States (Byrnes *et al.*, 2009; Kidd *et al.*, 2004, 2007; MacDougall *et al.*, 2007; Upton *et al.*, 2007). The infection rate of *C. gattii* among citizens and visitors of Vancouver Island is approximately 39 times higher then in Northern Australia, where *C. gattii* is endemic (Galanis *et al.*, 2008; Kidd *et al.*, 2004). It has been shown that more than 80% of the tested Vancouver Island residents have antibodies against *C. gattii*, whilst only 25% of Vancouver Island-based miners tested before the onset of the outbreak have such antibodies (Griffiths, 2006). Based on annual *C. gattii* infection rates, Chambers and colleagues (2008) suggested that exposure to *C. gattii* may be higher in certain popular tourist destinations on Vancouver Island, such as the surroundings of Nanaimo and Parksville. A number of apparently healthy tourists who visited Vancouver Island for a short period of time became infected with *C. gattii* (Georgi *et al.*, 2009; Levy *et al.*, 2007; Lindberg *et al.*, 2007; F. Hagen, K. Tintelnot and T. Boekhout, unpublished results). In a recent study by MacDougall and co-workers (2006), the median incubation time of *C. gattii* among residents and visitors to Vancouver Island was calculated to be between six and seven months. However, Lindberg and colleagues (2007) published a case report about a previously healthy Danish tourist who developed symptoms of cryptococcosis six weeks after he returned from a one week stay on Vancouver Island. This suggested a much shorter incubation time for the outbreak-specific genotype of *C. gattii* than was previously thought.

Here we challenge the generally believed supposition that *C. gattii* is a primary pathogen (Dixit *et al.*, 2009; Sorrell, 2001; Speed & Dunt, 1995) by describing a patient who visited the affected area of Vancouver Island for two weeks, one year before the onset of symptoms of cryptococcosis that she developed after treatment with corticosteroids. Detailed molecular typing demonstrated that she was infected by a *C. gattii* isolate of genotype AFLP6/VGII identical to those causing the outbreak.

Case report

In August 2007, a 42 year-old woman presented with two weeks of progressive headache. Eight months earlier, she was diagnosed with autoimmune haemolytic anaemia as her first manifestation of systemic lupus erythematosus (SLE), and at

that time treatment with prednisolone 60 mg QD PO was started. At the moment of her presentation the dose had been tapered to 17.5 mg QD. On examination, no nuchal rigidity or focal neurological deficit was seen. Magnetic Resonance Imaging (MRI) of the brain was normal. Lumbar puncture showed an elevated opening pressure of 50 cm H_2O and cerebrospinal fluid (CSF) showed predominantly lymphocytic pleocytosis (77 lymphocytes per µl) with normal levels of protein and glucose. Because of the immunocompromised state of the patient, an Indian ink stain was performed and was found positive for encapsulated yeast cells similar to *Cryptococcus* spp. CSF cryptococcal antigen was positive with a titre of 1:128, and *Cryptococcus* spp. was cultured from the CSF. Further blood tests on admission revealed severe lymphopenia with a $CD4^+$-count of 20/mm^3. A combined HIV-antibody and p24-antigen test was negative. Treatment with intravenous amphotericin B 0.7 mg/kg QD and 5-flucytosine 25 mg/kg QID was started and continued for two weeks, followed by fluconazole 400 mg QD PO for ten weeks. Thereafter, fluconazole was continued, as prophylaxis, in a dose of 200 mg QD PO until January 13th of 2009. Because of signs of elevated intracranial pressure, repeated lumbar punctures were performed at two and nine days after admission. On day nine, the cryptococcal antigen titer in the CSF had decreased from 1:128 at admission to 1:4 and the culture was negative. The headache resolved after the last puncture. To reduce the risk of recurrent cryptococcal infection prednisolone was replaced by azathioprine 125 mg QD. The patient was in good clinical condition and was discharged from the hospital without neurological sequelae. Follow-up, more than two years, was uncomplicated.

The cultured *Cryptococcus* isolate (CBS10866) was further genotyped using Amplified Fragment Length Polymorphism (AFLP) fingerprint analysis, mating-type analysis by PCR and Multi-Locus Sequence Typing (MLST) of seven unlinked nuclear loci (*CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1*, and *TEF1α*; GenBank accession numbers: FJ830387-FJ830393) (Boekhout *et al.*, 2001; Fraser *et al.*, 2005; Lindberg *et al.*, 2007). These molecular investigations revealed that this isolate belonged to *C. gattii* genotype AFLP6A/VGIIa, and was found to be fully identical to isolates representing the highly virulent genotype that cause the ongoing outbreak on Vancouver Island, Canada.

After the diagnosis of the *C. gattii* infection, the patient was questioned about her travel history. At the age of six, she moved from Australia to the Netherlands, she last visited Australia in 1989. In August 2006, she travelled to British Columbia and Alberta for one month, including two weeks on Vancouver Island where she spent most of the time in and around Victoria. In July 2007, she went for two weeks to Greece (Athens and Crete) and shortly after her return to the Netherlands she became ill, as we have described above.

Discussion

The case presented here shows that a latent infection by *C. gattii,* caused by an isolate that is identical to the Vancouver Island outbreak genotype AFLP6A/VGIIa, was activated during the period that the patient underwent a treatment with corticosteroids (prednisolone) in order to suppress the autoimmune hemolytic anemia that the patient developed five months after her visit to Vancouver Island. From the literature, a limited number of apparently dormant *C. gattii* infections are known. In 1986, Bottone and colleagues described a previously healthy female patient who travelled to Mexico and California, respectively five and thirteen years before the onset of the *C. gattii* serotype B infection. Dromer and colleagues (1992) presented a case of cryptococcosis in a 42-year-old apparently immunocompetent Cambodian male who lived for more than twenty years in France. Approximately one year after he returned from a 3.5-year stay in Gabon, he developed symptoms of tuberculosis which was treated with intensive four-drug chemotherapy; subsequently the patient developed cryptococcosis caused by *C. gattii* serotype B. However, the *C. gattii* isolates from these two cases were not investigated by molecular techniques and, therefore, the conclusion that both patients developed cryptococcosis caused by an activated dormant *C. gattii* infection remains speculative. Recent cases of apparent dormant *C. gattii* infections with the Vancouver Island outbreak genotype AFLP6/VGII involved subjects who visited or lived in the region colonized by *C. gattii*, four and up to fourteen years before symptoms of cryptococcosis appeared (MacDougall *et al.*, 2007). One of these patients had an underlying disease (cancer and kidney failure) prior to the development of cryptococcosis caused by the outbreak specific genotype of *C. gattii*. However, as both patients lived in areas where *C. gattii* has been found, a recently acquired *C. gattii* infection could not be entirely ruled out.

In Europe, autochthonous *C. gattii* infections are known to occur almost exclusively in the Mediterranean area, especially that of Greece, Italy and Spain. Several veterinary and clinical cases from this region were described and involved *C. gattii* infections in goats and humans (Baró *et al.*, 1997; Colom *et al.*, 2005; Montagna *et al.*, 1997; Velegraki *et al.*, 2001). Using molecular techniques, it was found that these serotype B *C. gattii* strains were genotype AFLP4/VGI (F. Hagen, T. Boekhout, A. Velegraki, M.F. Colom and J.M. Torres-Rodriguez, unpublished data). Two recent clinical *C. gattii* cases in Greece were found to be caused by strains with genotype AFLP6/VGII, however, these strains had a different MLST genotype than those causing the ongoing outbreak (Ngamskulrungroj *et al.*, 2009; F. Hagen, T. Boekhout and A. Velegraki, unpublished data). The fact that our patient was infected with *C. gattii* AFLP6A/VGIIa, genetically identical to that of the outbreak, strongly suggests that she was infected during her stay on Vancouver Island, rather than during her holiday in Crete, Greece.

Our observations and the cases discussed above indicate that apparently immunocompetent residents of, and visitors to, British Columbia and the Pacific Northwest may become silently infected with *C. gattii*. Clinicians and health practitioners must be aware of the fact that an immunosuppressive treatment may activate a latent *C. gattii* infection in previously immunocompetent residents and visitors to Vancouver

Island and the Pacific Northwest. Since a *C. gattii* infection is easily neglected in non-endemic regions, such as Europe, it is essential to obtain a detailed travel history when diagnostic features of cryptococcosis (e.g. persistent headache with or without fever, lethargy and personality changes) are observed and an immunosuppressive treatment is given or considered.

Given that the vast majority of tested Vancouver Island residents have antibodies against *C. gattii* (Griffiths, 2006), it is likely that more activated dormant infections with *C. gattii* AFLP6/VGII may appear in the future. It remains to be seen, however, how many visitors become infected during their stay on Vancouver Island or the Pacific Northwest. The current number of six infected tourists (Georgi *et al.*, 2009; Levy *et al.*, 2007; Lindberg *et al.*, 2007; F. Hagen, K. Tintelnot and T. Boekhout, unpublished results) indicates that this happens and, therefore, clinicians and health practitioners should be alert to the fact that activation as a consequence of a weakened immune status may occur long after the initial infection.

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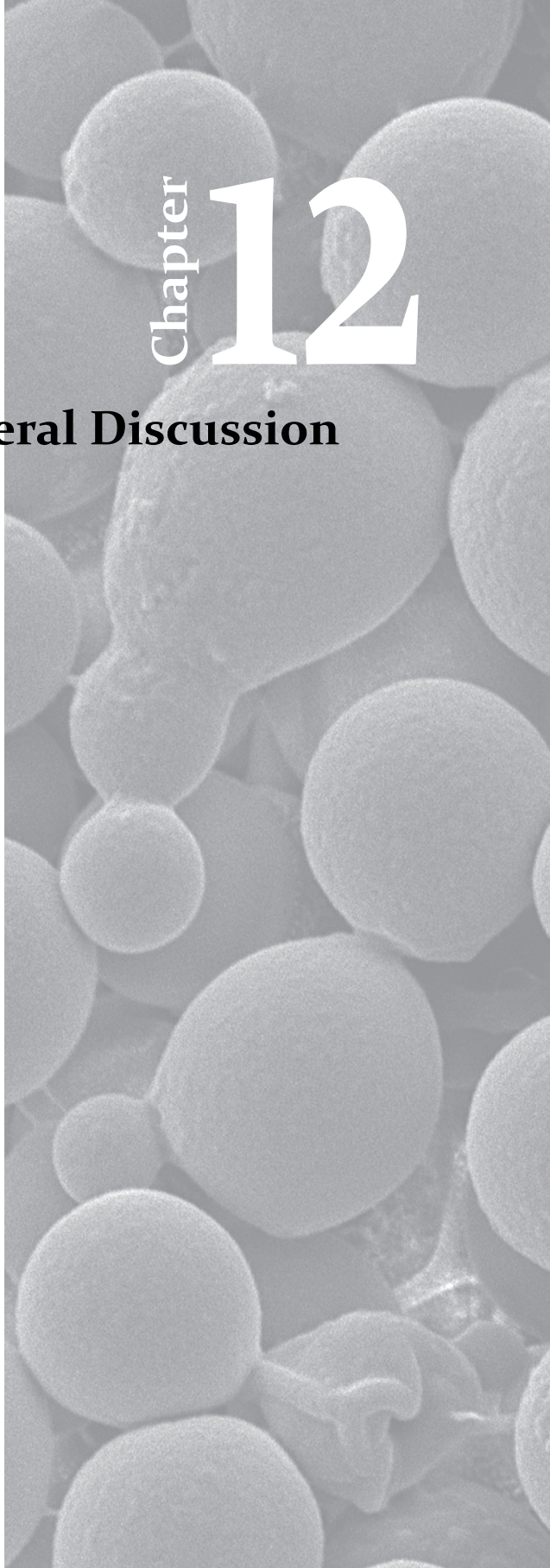
Chapter 12

Summary and General Discussion



Summary and General Discussion

Cryptococcus neoformans and *Cryptococcus gattii* are the leading cause of fungal meningitis worldwide. Until three decades ago both pathogenic basidiomycetous yeast species were, however, rarely encountered (Nielsen & Heitman, 2007). The onset of the HIV-pandemic during the early 1980s showed the deadly potential of the opportunist *C. neoformans* among HIV-infected individuals (Mitchell & Perfect, 1995). Recently it has been estimated that annually approximately one million HIV-positive patients develop cryptococcal meningitis, resulting in 625.000 fatalities. This made *C. neoformans* one of the leading causes of death, followed by tuberculosis, in HIV-patients from sub-Saharan Africa (Park *et al.*, 2009; Warkentien & Crum-Cianflone, 2010). However, not only immunocompromised individuals are threatened, as shown by unprecedented emergence of the primary pathogen *C. gattii* that causes the expanding outbreak in the temperate climate of British Columbia (Canada) and the Pacific Northwest (Kidd *et al.*, 2004).

The application of molecular biological techniques in the field of cryptococcal epidemiology revealed that previously observed epidemiological differences between *C. neoformans* and *C. gattii* are much less black and white than previously assumed. Within both species genotypes exist that infect immunocompetent or immunocompromised individuals. Examples are the occurrence of *C. gattii* as the cause of cryptococcosis among HIV-infected patients in Africa and *C. neoformans* variety *grubii* infections among apparently healthy Chinese individuals (Chen *et al.*, 2008; Litvintseva *et al.*, 2005). Subtle epidemiological differences seem to exist, and this is particularly visible between the current genotypes rather than between the species *C. neoformans* and *C. gattii*. Thus the advance of molecular biology has clearly shown that the current taxonomy of *C. neoformans* and *C. gattii* needs a thorough revision (this thesis; Bovers *et al.*, 2008a, 2008b; D'Souza *et al.*, 2011; Kwon-Chung & Varma, 2006; Ngamskulrungroj *et al.*, 2009).

The aim of this thesis was to investigate the epidemiology and population biology of the two major fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* and thereby provide additional support for a future taxonomic reappraisal of the species complex. Both species are investigated using a comprehensive set of molecular epidemiological tools combined with diverse phenotypic assays to further unravel the geographic and genetic origin of enhanced virulence.

This thesis consists of three sections: Section I contains the Chapters 2, 3 and 4 that focus on the epidemiology of *C. neoformans* in the Netherlands, Spain and Croatia, respectively. Section II includes Chapters 5, 6 and 7 that focus on the epidemiology of *C. gattii*. Section III comprises the Chapters 8, 9, 10 and 11 that are all dedicated to the population biological, epidemiological, and clinical aspects of the *C. gattii* outbreaks in North America.

Section I. Epidemiology of *Cryptococcus neoformans* in Croatia, the Netherlands and Spain

The epidemiology of *C. neoformans* has been regularly investigated since the onset of the HIV-pandemic in the early 1980s, either investigating the genetic diversity or antifungal susceptibility profiles of *C. neoformans* and these studies had a clinical epidemiological point of view (Baró *et al.*, 1999; Dromer *et al.*, 1996, 2007; Tintelnot *et al.*, 2001, 2004; Tortorano *et al.*, 1997; Van Elden *et al.*, 2000; Viviani *et al.*, 2006). A recently performed prospective epidemiological study showed differences between the population structure of North European and Mediterranean European *C. neoformans* isolates (Viviani *et al.*, 2006). To obtain a detailed insight into the epidemiology of this opportunistic pathogenic yeast in various European countries we conducted epidemiological studies in Croatia, the Netherlands and Spain that included antifungal susceptibility testing, mating- and serotyping assessment by PCR, and genotyping using AFLP and microsatellite typing (Chapters 2, 3 and 4).

The Croatian, Dutch and Spanish studies were performed using sets of isolates collected during 2005-2010, 1977-2007 and 1990-2007, respectively. In the Netherlands and Spain the incidence of cryptococcosis was higher among HIV-infected patients, namely 62.8% and 87.3%, respectively, but in Croatia a smaller proportion of the cryptococcosis patients was HIV-infected (33.3%). Previously conducted European studies observed a high number of HIV-infected individuals among cryptococcal infected patients that ranged from 68 to 77% (Dromer *et al.*, 2007; Tintelnot *et al.*, 2004; Viviani *et al.*, 2006). In Europe, the incidence of cryptococcal infections among immunocompetent patients has been poorly characterized. Two studies showed that 4% of the patients with cryptococcosis had no underlying disease (Dromer *et al.*, 2007; Tintelnot *et al.*, 2004). In the current Spanish study no immunocompetent patients were included, but in the Dutch cryptococcosis patient population nearly ten percent was immunocompetent and even two-third of the Croatian patients were found to be immunocompetent. The Croatian situation seems to be similar to that recently observed in several Asian countries where cryptococcosis is observed to occur among immunocompetent individuals, ranging from 19 to 92% of the patients with cryptococcosis (Chau *et al.*, 2010; Choi *et al.*, 2010; Day *et al.*, 2011; Liaw *et al.*, 2010; Zhu *et al.*, 2010). Interestingly, these Asian immunocompetent patients were found to be infected with *C. neoformans* var. *grubii*, which was also the case for 40% of the Croatian and for 83.3% of the Dutch immunocompetent cryptococcosis patients. Recent studies from South Korea and Vietnam observed a correlation between the immune status of the patient and the genotype of the isolated *C. neoformans* var. *grubii* strain (Choi *et al.*, 2010; Day *et al.*, 2011). However, a correlation between AFLP or microsatellite genotype and the clinical background of the patient was not observed for any of the current studied European populations.

In contrast to *C. neoformans* var. *grubii*, the epidemiology of *C. neoformans* var. *neoformans* has been poorly studied but it has been found that the latter is more prevalent in Europe and South America (Dromer *et al.*, 2007; Kwon-Chung & Bennett, 1984; Meyer *et al.*, 2010 ; Viviani *et al.*, 2006). In Europe, *C. neoformans* var. *neoformans*

(genotype AFLP2) is more frequently encountered in Mediterranean countries than in Northwestern Europe (Viviani *et al.*, 2006). This was further confirmed by our studies which showed that 13.1% of the Dutch patients were infected by genotype AFLP2 isolates, while this was 19.6% for Spanish and 33.3% of the Croatian patients, respectively. However, none of the current studies showed percentages close to that observed in Italy were 71% of the *C. neoformans* isolates were found to have a serotype D background (Tortorano *et al.*, 1997).

A higher prevalence of serotype AD hybrids between both *C. neoformans* varieties was observed in Europe when compared to other geographic localities (Kwon-Chung & Bennett, 1984). Recently, it was found that 19% of the patients from an European prospective study were infected with a serotype AD isolate (Viviani *et al.*, 2006). This is nearly equal to the percentage of patients from Croatia (20%), whereas in Madrid (Spain) this was much higher and here serotype AD hybrids caused 37.5% of the infections. In contrast, only 5.5% of the Dutch patients were found to be infected with the *C. neoformans* intervarietal hybrid. From this we conclude that infections with *C. neoformans* var. *neoformans* and the *C. neoformans* intervariety serotype AD hybrid occur more often in the European Mediterranean area if compared to Northwestern Europe. The Mediterranean area has a higher proportion of infections caused by *C. neoformans* var. *neoformans* and the *C. neoformans* intervariety serotype AD hybrid, when compared to the North Western area (Chapter 2, 3 and 4; Viviani *et al.*, 2006). Other epidemiological surveys from Brazil, China, South Korea, Taiwan and Vietnam revealed that serotype D isolates are rarely found, if not absent (Barreto de Oliveira *et al.*, 2004; Chau *et al.*, 2011; Choi *et al.*, 2010; Day *et al.*, 2011; Liaw *et al.*, 2010; Meyer *et al.*, 2010). The environmental niches of *C. neoformans* var. *neoformans* and the serotype AD hybrids in the Mediterranean area are yet unknown. Knowing the ecological niche may contribute to a better understanding of the high clinical prevalence of *C. neoformans* var. *neoformans* and the serotype AD hybrids in Mediterranean Europe. The application of microsatellite typing or MLST will be a valuable tool to further elucidate the genetic diversity of isolates with a serotype D background from Mediterranean Europe and other geographic regions. So far, multi-locus sequence typing (MLST) has not yet been applied on serotype D isolates and it will be laborious to perform such a technique on serotype AD hybrids due to the potentially presence of double alleles. A specific microsatellite typing approach for isolates with a serotype D background was recently developed and has so far only been applied to a small set of Dutch isolates. The results from this Dutch study are promising, thus this technique may be successfully applied on isolates from other localities (Chapter 2). Microsatellite typing has shown that epidemiological typing of serotype AB and AD hybrids is less laborious to perform than MLST. The former typing method easily discerns the background of serotype A (genotype AFLP1) and serotype D (genotype AFLP2) alleles among AD hybrids, while for MLST additional cloning steps are necessary to obtain a copy of one of each alleles from the hybrid genome (Chapter 2).

Microsatellite typing applied to *C. neoformans* var. *grubii* has shown that this approach is an excellent tool to discriminate isolates as well as to study their epidemiology (Illnait-Zaragozí *et al.*, 2010a, 2010b). Therefore, this technique was applied to a set of

Dutch cryptococcal isolates with a serotype A background. This study revealed that the genetic diversity observed in the Netherlands was higher than that occurring in Cuba (Chapter 2). The higher genetic diversity of the Dutch *C. neoformans* var. *grubii* population if compared to the Cuban populations may be due to one or more of the following. Firstly, the time span of the Dutch retrospectively study was longer. Secondly, the composition of the Dutch population is characterized by extensive immigration and includes people who came from former Dutch overseas colonies and immigrants from Northern Africa and Eastern Europe (CBS Statistics Netherlands, http://www.cbs.nl/, population data 1899-2010). It is likely that a proportion of the Dutch cryptococcosis patients are immigrants that acquired a subclinical infection with *C. neoformans* prior to immigration to the Netherlands, and this, in turn, may have contributed to the higher genetic diversity observed in the studied cohort. Thirdly, it has recently been shown that *C. neoformans* can produce *in vivo* diploid offspring from haploid parental isolates, either via cell fusion or endoreplication (Desnos-Ollivier *et al.*, 2010). Thus a further possibility is that dormant *C. neoformans* isolates become activated upon immune suppression and that during the course of infection and/or antifungal therapy *C. neoformans* cells fuse to form diploid cells that might be more resistant against antifungal compounds (Desnos-Ollivier *et al.*, 2010). The recent observation of the interspecies hybrids between *C. neoformans* and *C. gattii* that originated from clinical sources suggests that these interspecies hybrids may be formed *in vivo* (Bovers *et al.* 2006, 2008c). Taken together, microsatellite typing seems a powerful technique to investigate *C. neoformans* var. *grubii* and var. *neoformans* populations and this may provide better insights into the population structure and dynamics of isolates obtained from clinical, veterinary and environmental sources.

Antifungal susceptibility testing has frequently been applied on *C. neoformans*. The development of new antifungal drugs, such as the novel triazoles, and the reported increase in antifungal resistance, highlights the importance of monitoring the susceptibility profiles of clinical *C. neoformans* isolates. A global antifungal susceptibility study revealed that the observed resistance against amphotericin B, 5-flucytosine and fluconazole was less than one percent of the tested isolates (Pfaller *et al.*, 2005). Antifungal susceptibility testing of the Croatian, Dutch and Spanish isolates revealed that antifungal resistance is currently not a major problem as we found less than one percent of resistant isolates, similar to Pfaller and co-workers (2005). Several recently published antifungal susceptibility studies, including the studies presented in this thesis, showed that posaconazole, voriconazole and isavuconazole are promising candidates to substitute the conventional drugs since the latter have toxic side effects and needs to be administered in higher doses (Illnait-Zaragozí *et al.*, 2008; Pfaller *et al.*, 2005; Thompson *et al.*, 2009). Croatian and Dutch isolates that belong to *C. neoformans* var. *grubii* (AFLP1) or the serotype AD hybrid (AFLP3) were found to be less susceptible to fluconazole than *C. neoformans* var. *neoformans* (AFLP2) isolates. Next to this, the current studies showed that *C. neoformans* var. *neoformans* (genotype AFLP2) isolates had lower MIC values to amphotericin B. None of the three current studies revealed significant differences between patient categories versus antifungal susceptibility outcomes (Chapter 2, 3 and 4).

Section II. Genotypic diversity and antifungal susceptibility of *Cryptococcus gattii*

Until a decade ago, *Cryptococcus gattii* was rarely encountered as a cause of cryptococcosis outside the endemic localities in tropical and subtropical climate zones in Central Africa, Northern Australia, and South America (Kwon-Chung & Bennett, 1984; Springer & Chaturvedi, 2010). This changed due to the unprecedented emergence of an *C. gattii* outbreak at Vancouver Island (British Columbia, Canada) from where it further expanded to the mainland of Canada and the Pacific Northwest (Byrnes *et al.*, 2010; Datta *et al.*, 2009; Kidd *et al.*, 2004). An increase of cases in previously non-endemic areas has also been noticed (Springer & Chaturvedi *et al.*, 2010). This indicates that *C. gattii* might be, or has become more widespread than was thought before.

Cryptococcus gattii is increasingly found in Europe, and therefore we initiated an epidemiological survey to investigate *C. gattii* and atypical *C. neoformans* isolates that were isolated from European citizens, veterinary and environmental sources. This resulted in a set of more than 100 *C. gattii* isolates that was investigated using AFLP genotyping and MLST. The genetic relatedness between European isolates and those from other geographic localities was further investigated by the addition of a large set of globally collected *C. gattii* isolates (Chapter 5).

The oldest documented case of a European *C. gattii* infection was made by Curtis (1896), who described an infection in an otherwise healthy young man from Lille, France. Nevertheless, *C. gattii* infections remained exceptional in Europe for a long time, as highlighted by anecdotal reports such as that of a *C. gattii* infection in a young immunocompetent German citizen (Kohl *et al.*, 1985). Since a decade, *C. gattii* infections are on the rise in Europe but so far it remained unclear if these cases were due to autochthonous infections or related to travelling in endemic regions (Bodasing *et al.*, 2004; Grosse *et al.*, 2001; Hagen *et al.*, 2007; Velegraki *et al.*, 2001; Viviani *et al.*, 2006). Recent molecular epidemiological studies revealed that *C. gattii* is able to cause subclinical infections in humans and animals and that these infections were acquired at another continent (Chapter 11; Illnait-Zaragozí *et al.*, 2011). The current MLST study revealed that clinical isolates obtained from Northwestern European citizens mainly could be linked to *C. gattii* populations from other continents, while nearly all clinical and veterinary isolates from Mediterranean Europe were genetically closely related or genetically indistinguishable from environmental isolates obtained from this region (Chapter 5).

In Spain, several small *C. gattii* outbreaks among goats were reported and were found to belong to genotype AFLP4 (Baró *et al.*, 1998). An environmental sampling in the province of Apulia (Italy), initiated after the observation of an apparently autochthonous *C. gattii* infection, observed the presence of genotype AFLP4 isolates in environmental and veterinary samples (Montagna *et al.*, 1997). The recent observation of several veterinary and apparently autochthonous clinical cases in Spain were the basis to further investigate the occurrence of *C. gattii* in the Spanish environment (Chapter 6). Carob trees (*Ceratonia siliqua*) were found to harbor *C. gattii*. This tree

species is endemic in the Mediterranean region and its colonization can be considered as evidence for a Mediterranean niche of *C. gattii*. Several Mediterranean stone pines and one *Eucalyptus* tree yielded *C. gattii*. The latter tree species was previously thought to be the primary nice of *C. gattii* and it was believed that *C. gattii* dispersed globally due the export of Australian *Eucalyptus* trees and seedlings (Ellis & Pfeiffer, 1990). Therefore, the majority of European environmental surveys focused primarily on *Eucalyptus* trees, but this yielded only a few positive samples in Egypt and Italy (Mahmoud, 1999; Montagna *et al.*, 1997). However, growing evidence indicated that other tree species also harbored *C. gattii* (Springer & Chaturvedi, 2010). For example, a large environmental survey that was performed to monitor the expansion of the *C. gattii* outbreak in North America yielded many positive endemic tree species while *Eucalyptus* trees remained negative for *C. gattii* (Kidd *et al.*, 2007). This and other reports clearly showed that not *Eucalyptus* trees but plants, trees and their decaying products are the natural niche of *C. gattii* (Hagen & Boekhout, 2010; Springer & Chaturvedi, 2010).

When the Spanish environmental isolates were compared with those from clinical and veterinary origin it was observed that the majority of these isolates were genetically indistinguishable by AFLP fingerprinting and MLST (Figure 2 in Chapter 6). The identical genotypes of the Spanish *C. gattii* AFLP4 isolates strongly suggest that animals and humans with cryptococcosis acquired *C. gattii* from sources in their local environmental, such as trees. Interestingly, clinical and veterinary *C. gattii* isolates from those of the Western and Northwestern provinces of Spain were found to be genetically indistinguishable from the carob tree isolates that does not occur at these localities. Thus the ecological source for these *C. gattii* infections remains to be determined (Chapter 6).

Nearly all isolates from the European Mediterranean area formed a separate phylogenetic cluster that seems to have a clonal structure despite the presence of opposite mating-types (Figure 2 in Chapter 5). The presence of both mating-types within an apparently clonal population suggests that sexual recombination may occur. A similar observation was made based on a set of *C. gattii* AFLP4 isolates from Papua New Guinea (Campbell *et al.*, 2005). Here, it was hypothesized that inbreeding between genetically closely related isolates exist, but that no disturbance of the apparently clonal population structure occurs (Campbell *et al.*, 2005). This might also be the case within the European Mediterranean *C. gattii* AFLP4 population, but it remains remarkable that all mating-type **a** isolates from this Mediterranean cluster were found in Italy, while the Spanish isolates were all mating-type α which suggests a geographic barrier (Chapter 5).

Recombination was also observed within other genotypic groups of the *C. gattii* complex and supports previous observations (Chapter 5; Carter *et al.*, 2010; Ngamskulrungroj *et al.*, 2009). Besides regular mating occurring between strains with opposite mating-types **a** and α, it has been shown that same-sex reproduction of *C. neoformans* exist and that this mechanism might be the driving force behind the origin of the hypervirulent *C. gattii* AFLP6 outbreak in Canada (Fraser *et al.*, 2005; Wang & Lin, 2011). However, the occurrence of same-sex mating may conceal signals of recombination, which might be the case for the apparently clonal *C. gattii*

AFLP4 population structure in Mediterranean Europe. This strongly suggests that recombination might occur here but that it remains invisible, as suggested in several Australian studies (Campbell *et al.*, 2005; Saul *et al.*, 2008).

Within Europe, *C. gattii* AFLP4 has only been found in the European Mediterranean environment. This suggests that geographical boundaries exists, either caused by the climate that prevails in this region and/or the Pyrenean and Alps mountains that separates the Iberian Peninsula and Italy from the European mainland. This occurrence in a geographically limited region is further strengthened by the observation that a proportion of clinical case from Northwestern Europe could be linked to other endemic localities, such as Brazil, California, and the African continent, while clinical isolates from Mediterranean Europe are genetically closely related to environmental and veterinary isolates obtained from that region.

MLST analysis based on ten unlinked nuclear loci and the seven loci datasets according to Fraser *et al.* (2005) and Meyer *et al.* (2009) showed the presence of five monophyletic clusters within *C. gattii* (Chapter 5). This supports previous studies that have used other molecular biological typing techniques, such as M13 PCR fingerprinting and AFLP fingerprint analysis (Boekhout *et al.*, 2001; Meyer *et al.*, 2009). MLST is a valuable tool to trace the geographic origin of clinical and veterinary isolates. From these studies we conclude that clinical *C. gattii* infections from Northwestern Europe are mainly acquired outside the European continent, for example during visiting an endemic region like Vancouver Island, or before migration to Europe from endemic regions in Africa and South America. Reactivation of dormant *C. gattii* infections upon immune suppression seems to occur more often than was assumed before, and this indicates that *C. gattii* is more likely to be an opportunistic rather than a primary pathogen, as has been recently suggested (Chapter 11; MacDougall *et al.*, 2011).

Despite the emergence of *C. gattii* during the past decade there is a lack of in-depth studies on the susceptibility of *C. gattii* strains against conventional and new antifungal compounds (Datta *et al.*, 2009). Correlations between susceptibility profiles and the genotypic background of the *C. gattii* isolates have rarely been studied and, so far, only with relatively low number of isolates (Iqbal *et al.*, 2010). Therefore, we have compared the antifungal susceptibility profiles of 350 globally collected *C. gattii* isolates (Chapter 7). These isolates were tested for the four clinically used antifungal compounds amphotericin B, 5-flucytosine, fluconazole and itraconazole, as well as for the three novel triazoles voriconazole, posaconazole and isavuconazole. This revealed that the conventional antifungal drugs amphotericin B, 5-flucytosine and fluconazole had the least activity against *C. gattii* strains when compared to itraconazole and the novel triazoles. The observation that 5-flucytosine and fluconazole had a decreased antifungal activity against *C. gattii*, as well as against its sibling *C. neoformans*, is in line with previous findings (Gomez-Lopez *et al.*, 2008; Illnait-Zaragozí *et al.*, 2008; Iqbal *et al.*, 2010; Pfaller *et al.*, 2005; Thompson *et al.*, 2009; Trilles *et al.*, 2004). Although breakpoints for *C. gattii* are not yet defined, 'surrogate breakpoints' as introduced by Pfaller and co-workers (2005) for amphotericin B, 5-flucytosine and fluconazole were used for its sibling *C. neoformans*. All *C. gattii* isolates were (highly) susceptible when these breakpoints were used, with minimal inhibitory concentrations (MICs) of ≤1 μg/

ml for amphotericin B, as previously observed (Iqbal *et al.*, 2010; Thompson *et al.*, 2009; Trilles *et al.*, 2004). However, a decreased *in vitro* susceptibility (≥16 µg/ml) against fluconazole was observed for 7.4% of the *C. gattii* isolates, and one of these 26 isolates was found to be resistant (≥64 µg/ml). With respect to 5-flucytosine, 24.3% fell within the group of 'decreased susceptible' (≥8 µg/ml) and 2.6% fell into the category of 'highly resistant' isolates (≥32 µg/ml) (Chapter 7).

In the present study the majority of isolates belonged to genotype AFLP4 (39.1%) and AFLP6 (48.3%), while the remaining 12.6% of the *C. gattii* isolates fell into one of the minor genotypes (AFLP5, 7-10). The preponderance towards *C. gattii* strains with an AFLP4 or AFLP6 genotype profile may be caused by a non-representative selection of isolates due to the inclusion of preserved strains from well-studied geographic localities such as Australia and the *C. gattii* outbreak region in North America where isolates with AFLP4 and AFLP6 are more frequently isolated (Iqbal *et al.*, 2010; Kidd *et al.*, 2004; Springer & Chaturvedi, 2010). Investigated *C. gattii* isolates from the less studied regions Africa, Asia and Europe followed the trend that strains with an AFLP4 genotype profile were more frequently isolated than others (Chapter 5; Springer & Chaturvedi, 2010).

The present study investigated the influence of the genetic background and the geographic origin of *C. gattii* isolates on the antifungal susceptibility outcome. A significant ($P<0.05$) higher geometric mean MIC for six of the seven antifungal compounds was observed for clinical *C. gattii* AFLP6 isolates versus genotype AFLP4 isolates (Chapter 7). Strikingly, the latter genotypic group had low geometric mean MICs of 1.401 and 2.467 µg/ml for 5-flucytosine and fluconazole, respectively, compared to clinical isolates within genotype AFLP6 (geometric mean MICs of 4.961 and 5.638, respectively). Similar observations were recently observed for a set of 43 North American *C. gattii* isolates (Iqbal *et al.*, 2010). In the current study, fluconazole and 5-flucytosine had the lowest activity against *C. gattii* strains. Itraconazole, which has been used to treat cryptococcosis, had a MIC_{90} of only 0.25 µg/ml, which is one or two $\log_2$ dilutions lower than found previously by others (Iqbal *et al.*, 2010; Trilles *et al.*, 2004). Isavuconazole, posaconazole, and voriconazole demonstrated excellent potency against each isolate and all AFLP genotypes, including isolates with a reduced susceptibility to fluconazole. We conclude that amphotericin B, itraconazole, posaconazole, voriconazole and isavuconazole were the most active antifungal compounds with *in vitro* activity against *C. gattii* isolates. Isavuconazole, with its attractive pharmacological and toxicological properties and availability in oral and parenteral formulations, may be a welcome addition to the clinician's antifungal armamentarium to fight this pathogen (Chapter 7).

Section III. Hypervirulence of *Cryptococcus gattii* – The Vancouver Island outbreak

During the past decade several fungal outbreaks emerged that have affected humans, mammals, reptiles and insects. The majority of these outbreaks are caused by previously unknown fungal species, such as *Geomyces destructans* that is the cause of white-nose-syndrome in bats, *Batrachochytrium dendrobatidis* known as the frog-killing fungus, *Nosema ceranae* that is involved in massive honeybee colony collapses, and *Cryptococcus gattii* that is the cause of several outbreaks in North America, South America and Western Australia and has affected hundreds of humans and animals (Blehert *et al.*, 2009; Byrnes *et al.*, 2010; Carriconde *et al.*, 2011; Higes *et al.*, 2008; James *et al.*, 2009; Kidd *et al.*, 2004).

Cryptococcus gattii gained attention a decade ago due to an unprecedented outbreak that emerged at Vancouver Island (British Columbia, Canada) and that subsequently expanded to the mainland of the Pacific Northwest (Kidd *et al.*, 2004; Datta *et al.*, 2009). The cryptococcal outbreak at Vancouver Island was caused by an until then rarely seen genotype of *C. gattii* known as AFLP6 (Kidd *et al.*, 2004). Recent studies, however, have shown that *C. gattii* AFLP6 was also involved in outbreaks among captivated psittacine birds in Brazil, among over 100 sheep in Western Australia and it has been noted that a large proportion of *C. gattii* AFLP6 infections occur among children in the province of Pára, Brazil (Carriconde *et al.*, 2011; Costa *et al.*, 2009; Raso *et al.*, 2004).

Fraser and co-workers (2005) made a first attempt to trace the origin of the Vancouver Island *C. gattii* outbreak for which they used MLST. They concluded that the outbreak was caused by a novel hypervirulent genotype that originated most likely from a same-sex mating event between two avirulent Australian *C. gattii* AFLP6 strains (Fraser *et al.*, 2005). A second study that appeared soon thereafter concluded that the global population structure of *C. gattii* AFLP6 is highly clonal and, therefore, it was assumed that the origin of the outbreak could originated from all the investigated populations (Kidd *et al.*, 2005). Two other studies pointed towards a South American origin of the Vancouver Island *C. gattii* AFLP6 outbreak, although an African or European origin could not be ruled out completely (Byrnes *et al.*, 2010; Ngamskulrungroj *et al.*, 2009).

To solve this controversy on the origin of the various *C. gattii* AFLP6 outbreaks, we investigated a collection of *C. gattii* AFLP6 strains from Africa, Australasia, Europe, North and South America using four different molecular epidemiological approaches. This included a MLST scheme based on sequence characterized AFLP regions (SCAR-MLST), AFLP fingerprinting, a novel microsatellite typing scheme and a re-analysis of MLST data published previously by Fraser *et al.* (2005) and Byrnes *et al.* (2010) (Chapter 8). All four datasets revealed that South America has the highest genetic diversity and the largest mean effective population size compared to Australasia and North America (Supplementary Table 4 in Chapter 8). This is in agreement with previous studies that showed that *C. gattii* is genetically diverse in South American (Meyer *et al.*, 2003; Ngamskulrungroj *et al.*, 2009; Trilles *et al.*, 2003).

The SCAR-MLST dataset was subsequently used to infer the coalescence gene genealogy and to time-scale the diversification of *C. gattii* AFLP6. This analysis showed that South America is the geographic origin of the global *C. gattii* AFLP6 population. Interestingly, the most ancestral lineage was represented by a strain that was isolated from a tree in the pristine Brazilian Amazon rainforest. From this ancestral lineage, two lineages emerged with an African and South American origin and the third and youngest lineage further branched into 28 lineages including lineages, that are involved in currently occurring outbreaks in North America, South America and Western Australia (Figure 1 in Chapter 8). The time to the most recent common ancestor was estimated to be approximately 30,000 years before present (ybp) and the Vancouver Island *C. gattii* AFLP6A outbreak (H1) lineage emerged around 2,500 ybp. Other outbreak-related lineages were found to be older. The lineages represented by strains isolated from the outbreak among psittacine birds (H2) and those isolated from an outbreak among sheep in Western Australia (H3) emerged approximately 9,300 ybp. A fascinating observation is that the latter lineage also contained strains from the Vancouver Island outbreak with the so-called 'minor genotype' AFLP6B and the novel genotype AFLP6C that is involved in a recently emerged outbreak in the Pacific Northwest (Byrnes *et al.*, 2010; Datta *et al.*, 2009; Kidd *et al.*, 2004).

Several epidemiological studies, including ours, observed that both mating-type **a** and α strains are present within the European and South American *C. gattii* AFLP6 populations, while no mating-type **a** strains have been found so far in Africa and North America, and only recently mating-type **a** strains were found in Australia (Chapter 7; Carriconde *et al.*, 2011; Byrnes *et al.*, 2010; Fraser *et al.*, 2005; Kidd *et al.*, 2004). The presence of both mating-types in nearly equal amounts in the SA population suggests that recombination events are likely to occur in this locale. However, it has been found that South American mating-type **a** strains are less fertile than its mating-type α counterpart (Ngamskulrungroj *et al.*, 2008). Besides that, same-sex reproduction exists as a mode of reproduction for *C. gattii* and its sibling *C. neoformans* and was previously proposed as the cause of the Vancouver Island outbreak genotype (Fraser *et al.*, 2005; Lin *et al.*, 2005, 2007, 2009). When the existence of recombination in the five *C. gattii* AFLP6 populations was further explored using different approaches (i.e. Ancestral Recombination Graph and CASS) it was found that the amount of recombination differed widely per population, but that the recombination signal was disproportionally high in South America. The fact that the ancestral lineage had a South American origin and that at this locality both mating-types occur in nearly equal proportions suggest that the hypervirulent lineage may have derived from regular mating events rather than by same-sex.

We propose that the observed recombination might be the driving force behind the enhanced virulence of outbreak-related lineages. The virulence potential of *C. gattii* AFLP6 strains was further investigated by a macrophage assay and a murine pathogenicity model using strains from the Vancouver Island outbreak that fell into genotype AFLP6A and a set of isolates with genotype AFLP6B that originated from the outbreak locality as well as from other geographic regions. This exploratory study revealed that *C. gattii* AFLP6A strains from the Vancouver Island outbreak were

significantly more virulent in a murine and macrophage model (Chapter 9). Next to this, it was shown that a correlation between intracellular macrophage proliferation of *C. gattii* and its virulence potential in the mice pathogenicity model exist (Chapter 9). However, recent observations showed that strains that are avirulent in a mice pathogenicity model can proliferate inside macrophages (Chapter 8). Other recent studies confirmed the difference in virulence between genotype AFLP6A and AFLP6B strains using a similar approach or solely the established murine model (Byrnes *et al.*, 2010; Ngamskulrungroj *et al.*, 2008). The recently emerging outbreak of *C. gattii* AFLP6C in the Pacific Northwest was found to have a similar virulence potential as the strains from the Vancouver Island outbreak with genotype AFLP6A (Byrnes *et al.*, 2010).

To elucidate the virulence potential of *C. gattii* AFLP6 strains not involved in any of the outbreaks, we investigated a selection of strains using BALB/c mice and J774 macrophage pathogenicity assays (Chapter 8). This revealed that two South American genotype AFLP6B strains from the ancestral lineages in the coalescence gene genealogy were highly virulent, but not as virulent as strains involved in the Vancouver Island and the Pacific Northwest outbreaks (Figure 2 in Chapter 8; Chapter 9; Byrnes *et al.*, 2010). We conclude that hypervirulence is not restricted to lineages involved in outbreaks, but that this trait was present among several non-outbreak related lineages, including the most ancestral lineage. Next to this it was observed that hypo- and hypervirulence traits exists next to each other in lineages that contain genetically indistinguishable strains, these include the Vancouver Island outbreak genotype AFLP6A lineage (Figure 1 in Chapter 8).

The genetic base for the enhanced virulence of *C. gattii* AFLP6A outbreak strains versus that of genotype AFLP6B strains was further explored using an expression micro-array approach revealing that the mitochondrial genome was upregulated for the outbreak strains when compared to that of non-outbreak related strains (Chapter 9). The involvement of mitochondria in the enhanced virulence of the Vancouver Island outbreak strains was subsequently strengthened by the observation that these strains exhibited a tubular mitochondrial morphology. Genes involved in the regulation of the mitochondrial morphology were found to be upregulated in *C. gattii* AFLP6A outbreak strains, thus supporting the involvement of tubular mitochondria in an enhanced virulence trait (Chapter 9). However, the role of mitochondria in fungal virulence remains to be clarified. Until recently a link between virulence and mitochondria was only observed for one other fungus, the basidiomycetous plant pathogen *Heterobasidion annosum* (Olson & Stenlid, 2001). Since mitochondria are involved in hypoxia and the oxidative stress response it seems likely that their genomes are under high selection pressure and accumulated mutations may have contributed to enhanced cryptococcal virulence (Chapter 9; Ingavale *et al.*, 2008; Lynch *et al.*, 2008). Our observations indicate that *C. gattii* AFLP6A strains can persist inside macrophages and that this is probably due to their ability to form tubular mitochondria in order to efficiently repair damage of the mitochondrial genome after exposure to oxidative stress and/or hypoxia while inside the phagosome (Chapter 9).

Smaller *C. gattii* AFLP6 outbreaks have been documented at other localities, including those among sheep in Western Australia since 1993, among psittacine birds in Brazil and infections among children from Pára, Brazil (Carriconde *et al.*, 2011; Costa *et al.*, 2009; Raso *et al.*, 2004). At the same time, *C. gattii* AFLP4 emerged in Mediterranean Europe where it caused several small outbreaks among goats (Chapter 6; Baró *et al.*, 1997). These examples show that *C. gattii* is moving towards temperate climate zones. Strikingly enough these outbreak localities are all situated in climate zones with a Mediterranean sea climate according to the recently updated Köppen-Geigen climate classification system (Peel *et al.*, 2007). The hypothesis of a sudden emergence of a single hypervirulent lineage that may have given rise to the various outbreaks can be ruled out since different lineages are involved among the occurred outbreaks (Chapter 8).

The majority of the *C. gattii* AFLP6A strains of the Vancouver Island outbreak genotype are hypervirulent, but exceptions have been observed. A clinical strain isolated during the early 1970s from a patient in Seattle (USA) was found to be attenuated in its virulence potential (Chapter 9; Fraser *et al.*, 2005). Similarly, an environmental *C. gattii* AFLP6A strain cultured in the 1990s from an *Eucalyptus* tree in California was found to be genetically similar to the Vancouver Island outbreak lineage, but was avirulent in mice and macrophage pathogenicity assays (Chapter 9). A recently performed study revealed that 64.9% of the Vancouver Island residents and 17.6% of the miners, sampled between 1982-1984, had antibodies against *C. gattii* (Griffiths, 2006). These data together clearly indicate that the Vancouver Island outbreak lineage was already present in the local environment decades before the onset of the outbreak.

The emergence of several *C. gattii* outbreaks at various localities may be related to one or more recent developments, such as a genetic event like recombination or a change in the environmental conditions. It is possible that global warming plays a key role in the emergence of the outbreaks in Mediterranean Europe, North America and Western Australia since they all appeared during the same period (Chapter 8; Levitus *et al.*, 2001). Studies on climate change velocity showed that temperate and Mediterranean climate zones belong to the groups with the highest velocity in temperature change (Loarie *et al.*, 2009). Thus an increase in the average annual temperature might influence the emergence and severity of pathogenic microorganisms in temperate climate zones (Harvell *et al.*, 2002). The increase of global temperatures and factors that are directly related to this, such as precipitation, humidity, air and water temperature, has changed the distribution patterns of a variety of pathogenic microorganisms affecting plants and animals (Harvell *et al.*, 2002; Jones *et al.*, 2008) and it seems likely that this is also true for *C. gattii* AFLP6 (Chapter 8).

Coalescence gene genealogy analysis showed that the oldest lineages originated from Brazil (Chapter 8). Approximately 22,000 ybp a lineage dispersed to Africa, followed by a massive dispersal and recombination event approximately 9,000 ybp that introduced *C. gattii* AFLP6 into Australasia, Europe and North America. Many lineages descended from this global dispersal event. Most of the South American lineages emerged during the past 6,000 to 7,000 years and were formed by a high rate of recombination events which may be the driving force behind the genetic diversification

at this continent. Long distance dispersal may have contributed to intercontinental dispersal of *C. gattii* AFLP6. Unusual and extreme weather events may contribute to the dispersal of microbial organisms (Brown & Hovmøller, 2002; Griffin, 2007). Aerial movements of human pathogenic fungi have not been investigated at a large scale, but it has been shown that fungi are present in dust storm samples and this might be a possible dispersal mechanism for the global distribution of *C. gattii* (Griffin, 2007). Nevertheless, the mode of long distance dispersal of *C. gattii* AFLP6 remains an enigma.

Since the onset of the Vancouver Island outbreak hundreds of previously healthy humans and animals living in the affected area developed cryptococcosis (Datta *et al.*, 2009). However, tourists visiting the endemic area of Vancouver Island are at risk as well. It has repeatedly been shown that during short visits to endemic areas, such as Vancouver Island, tourists acquire cryptococcosis (Chapter 10 and 11; Chambers *et al.*, 2008; Georgi *et al.*, 2009; Goldman *et al.*, 2010; Levy *et al.*, 2007). This thesis describes two case reports on *C. gattii* infections in two patients who acquired cryptococcosis during a short stay on Vancouver Island (Chapter 10 and 11). The first reported case concerned a tourist who developed cryptococcosis shortly after returning from Vancouver Island. The patient was a 51-year old HIV-negative, apparently immunocompromised Danish man, who was under treatment with a non-steroid anti-inflammatory drug. Six weeks before admission he returned from a three-week holiday at Vancouver Island, this highlights the short incubation time to develop clinical signs of a *C. gattii* infection (Chapter 10). In Chapter 11, a 42-year old female Dutch patient is described who developed cryptococcosis eight months after she was diagnosed with autoimmune haemolytic anaemia as her first manifestation of systemic lupus erythematosus (SLE). She was treated with prednisolone to suppress SLE, which, however, had activated the dormant cryptococcal infection. Extensive molecular research using AFLP fingerprinting and MLST showed that both patients were infected with *C. gattii* AFLP6A that was genetically indistinguishable from the strains involved in the Vancouver Island outbreak (Chapters 10 and 11). The case of the Danish patient showed the highly virulent phenotype of the *C. gattii* AFLP6A outbreak lineage, with an incubation time of only six weeks. In contrast, the case of the Dutch patient showed that *C. gattii* is able to cause a dormant infection that can be activated upon immune-suppressive therapy. This is described here for the first time, but was known for *C. neoformans*. The two previously described cases of latent *C. gattii* infections lack detailed molecular analyses of the causative agent. Thus the conclusions that such patients developed cryptococcosis caused by an activated dormant *C. gattii* infection remains highly speculative (Bottone *et al.*, 1986; Dromer *et al.*, 1992). The case of the Dutch patient highlights the possibility of dormant *C. gattii* AFLP6A infections among immunocompetent individuals that is especially important for residents of the affected outbreak region. The vast majority of tested Vancouver Island residents has antibodies against *C. gattii* and, therefore, it is likely that they develop cryptococcosis in the future as a result of immune-suppressive therapy (Chapter 11).

Concluding remarks

This thesis has provided additional support that epidemiological differences between *C. neoformans* and *C. gattii* are less stringent than was thought before. However, phylogenetic analyses based on data obtained by different molecular approaches shows that both species consist of monophyletic clusters that may correlate with immune-status of the patients, especially within the *C. gattii* complex. The occurrence of monophyletic clusters in *C. neoformans* and *C. gattii* advocates for a taxonomic reappraisal of both species. This is, however, currently the subject of debate among cryptococcal researchers and clinicians due to the fact that a significant biochemical, ecological and/or epidemiological difference between these cryptic species is not yet observed. The observation that *C. gattii* and *C. neoformans* can both infect immunocompromised and immunocompetent individuals indicates that subtle genetic and phenotypic differences exist between these species. Such an observation was made for *C. gattii* genotypes involved in the Vancouver Island outbreak and this may also occur within other genotypic lineages. Future research should focus on the search for easy-to-use genetic or phenotypic assays to differentiate the cryptic species. State-of-the-art whole genome sequencing approaches and large-scale population studies will further clarify the complex interaction between the host immune system and that of the various cryptic species within the *C. neoformans*/*C. gattii* complex.

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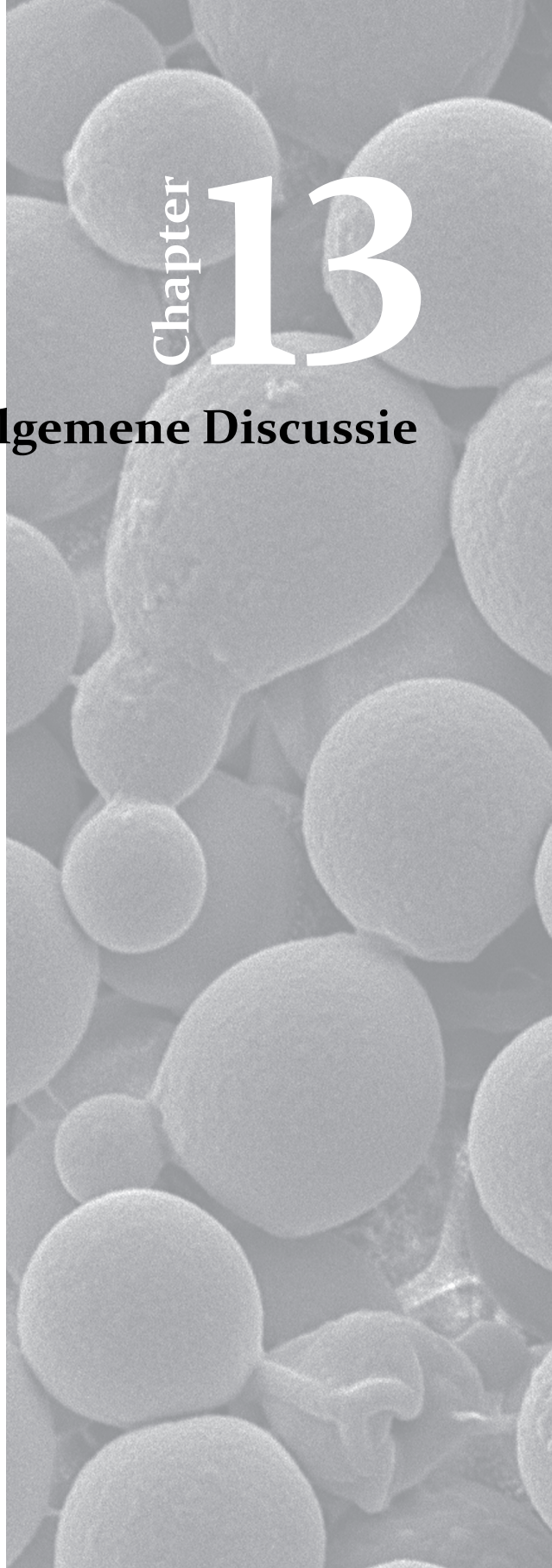
Chapter 13

Samenvatting en Algemene Discussie



Samenvatting en Algemene Discussie

Cryptococcus neoformans en *Cryptococcus gattii* zijn wereldwijd de meest voorkomende veroorzakers van schimmelgerelateerde meningitis. Tot drie decennia geleden waren deze pathogene basidiomycete gisten slechts zelden betrokken bij infecties. De opkomst van de HIV-pandemie aan het begin van de jaren 1980 legden het dodelijke potentieel bloot van *C. neoformans*, deze gist bleek hoofdzakelijk infecties te veroorzaken in personen met een verzwakt immuunsysteem, zoals HIV-besmette individuen. In een recente studie is berekend dat jaarlijks ongeveer één miljoen HIV-geïnfecteerde patiënten meningitis ontwikkelen wat veroorzaakt wordt door *C. neoformans*, circa 625.000 van deze patiënten overleven deze infectie niet. Hiermee zijn *C. neoformans* infecties, gevolgd door tuberculose, één van de hoofdoorzaken van sterfte onder Afrikaanse HIV-patiënten. Echter, niet alleen individuen met een verzwakte weerstand kunnen *Cryptococcus*-infecties oplopen, dit is eens en temeer duidelijk geworden door de plotselinge opkomst van infecties met de voorheen tropische gist *C. gattii*. Deze gist is de oorzaak van een zich nog steeds verder verspreidende uitbraak in British Columbia (Canada) en de Pacific Northwest (Verenigde Staten) waarbij gezonde personen de infectie oplopen terwijl personen met een verzwakt immuunsysteem ogenschijnlijk geen gevaar lopen.

De toepassing van moleculair biologische technieken in het aandachtsgebied van de *Cryptococcus* epidemiologie heeft aangetoond dat veronderstelde epidemiologische verschillen tussen *C. neoformans* en *C. gattii* veel minder zwart-wit zijn dan voorheen werd aangenomen. Beiden soorten bevatten genotypen die in staat zijn om gezonde (immuuncompetente) of individuen met een verzwakte weerstand (immuundeficiëntie) te infecteren. Voorbeelden hiervan zijn *C. gattii* als de veroorzaker van cryptococcosis onder HIV-geïnfecteerde patiënten in Afrika, en *C. neoformans* var. *grubii* infecties bij immuuncompetente Chinese patiënten. Subtiele epidemiologische verschillen lijken aanwezig te zijn, dit is vooral duidelijk wanneer de verschillende genotypen binnen *C. neoformans* en *C. gattii* met elkaar vergeleken worden. Deze verschillen zijn echter moeilijk waarneembaar wanneer enkel *C. neoformans* met *C. gattii* wordt vergeleken. De opkomst van de moleculaire biologie heeft duidelijk aangetoond dat de huidige taxonomische indeling van *C. neoformans* en *C. gattii* grondig moet worden herzien.

Het doel van het onderzoek beschreven in dit proefschrift was om de verschillen in epidemiologie en populatiebiologie van de twee ziekteverwekkende gisten *C. neoformans* en *C. gattii* te bestuderen en zodoende meer bewijs te leveren om in de nabije toekomst de taxonomische indeling te herzien. Beide gistsoorten werden uitgebreid onderzocht door middel van moleculair biologische technieken in combinatie met microbiologische tests waardoor epidemiologische verschillen verder ontrafeld kunnen worden. Dit proefschrift bestaat uit drie secties: Sectie I bevat de hoofdstukken 2, 3 en 4 en focussen respectievelijk op de epidemiologie van *C. neoformans* in Nederland, Spanje en Kroatië. Sectie II bevat de hoofdstukken 5, 6 en 7 en beschrijven het onderzoek verricht aan *C. gattii*. Sectie III bevat de hoofdstukken 8 tot en met 11 en zijn geheel gewijd aan de epidemiologie, populatiebiologie en klinische aspecten van de *C. gattii* uitbraken in Noord-Amerika.

Sectie I. Epidemiologie van *Cryptococcus neoformans* in Kroatië, Nederland en Spanje

Sinds de opkomst van *C. neoformans* infecties aan het begin van de jaren 1980 is de epidemiologie van deze gist met grootte regelmaat bestudeerd. Deze studies onderzochten vooral de genetische variatie of de werking van schimmeldodende middelen, maar bovenal focusseerde deze studies zich op de klinische achtergrond van de infecties. Een recente Europese studie heeft aangetoond dat er grote verschillen zijn in het voorkomen van de verschillende *C. neoformans* genotypen in Noordwest Europa ten opzichte van de Mediterrane landen. Om dit nader te bestuderen is de Nederlandse *C. neoformans* populatie vergeleken met dat van Kroatië en Spanje. Hiervoor is gebruik gemaakt van de moleculair biologische technieken wat het mogelijk maakte om de *C. neoformans* genotypen tussen de landen onderling te vergelijken, alsmede eventuele verschillen tussen de genotypen voor wat betreft de gevoeligheid tegen een set van zeven schimmeldodende middelen.

In Nederland en Spanje kwamen *Cryptococcus*-infecties vaker voor bij HIV-geïnfecteerde individuen (respectievelijk 62.8% en 87.3%). Terwijl in Kroatië slechts bij 33.3% van de onderzochte *Cryptococcus*-infecties de patiënt een verzwakt immuunsysteem had. Andere Europese studies rapporteerde dat 68 tot 77% van *Cryptococcus*-infecties voor kwam bij personen met een verstoord afweersysteem. Er is weinig onderzoek verricht naar cryptococcosis bij patiënten waarvan het immuunsysteem ogenschijnlijk normaal functioneert. Slechts twee studies merkte op dat een klein gedeelte van de onderzochte cryptococcosis patiënten (4%) een ogenschijnlijk goed werkend afweersysteem had. In de groep Spaanse patiënten werd bij alle patiënten een verstoord afweermechanisme aangetroffen, terwijl in de groep van Nederlandse en Kroatische cryptococcosis patiënten respectievelijk 10% en 33% van hen een normaal functionerend immuunsysteem bleek te hebben (Hoofdstuk 2, 3 en 4). Enkele recente Aziatische studies hebben aangetoond dat cryptococcosis daar voornamelijk voorkomt bij individuen met een ogenschijnlijk goed werkend immuunsysteem. Een interessante observatie is dat de Aziatische *Cryptococcus*-infecties werden veroorzaakt door *C. neoformans* var. *grubii*, wat ook in 40% van de Nederlandse en 83% van de Kroatische infecties het geval was. Epidemiologische studies uitgevoerd in Vietnam en Zuid-Korea hebben aangetoond dat er een verband is tussen het genotype van de gekweekte *C. neoformans* var. *grubii* stam en de klinische achtergrond van de patiënt waarbij deze stam geïsoleerd is. Echter, eenzelfde correlatie kon niet worden vastgesteld bij de Kroatische, Nederlandse en Spaanse epidemiologische studies (Hoofdstuk 2, 3 en 4).

In tegenstelling tot *C. neoformans* var. *grubii* (serotype A) is de epidemiologie van *C. neoformans* var. *neoformans* (serotype D) maar matig bestudeerd. Wel is bekend dat laatstgenoemde vaker voorkomt bij Europese en Zuid-Amerikaanse patiënten. Binnen Europa is gebleken dat *C. neoformans* var. *neoformans* in grote mate infecties

veroorzaakt in de Mediterrane landen en in mindere mate in het Noordwestelijke gedeelte. Dit werd bevestigd door onze studies die aantoonde dat ruim 13% van de Nederlandse cryptococcosis-patiënten geïnfecteerd was geraakt met *C. neoformans* var. *neoformans*, tegen bijna 20% van de Spaanse en ruim 33% van de Kroatische patiënten (Hoofdstuk 2, 3 en 4).

Het is bekend dat er in Europa relatief veel infecties voorkomen met de zogenaamde 'serotype AD hybriden' (een kruising tussen *C. neoformans* var. *grubii* en var. *neoformans*). Een recente Europese studie toonde aan dat 19% van de onderzochte patiënten dit type *Cryptococcus*-infectie had. Dit is bijna gelijk aan het aantal 'serotype AD'-infecties in Kroatië waar 20% van de infecties door deze soort werden veroorzaakt (Hoofdstuk 4). De Spaanse studie overtrof dit percentage, hier bleek ruim 37% van de patiënten met de 'serotype AD hybride' cryptococ geïnfecteerd te zijn (Hoofdstuk 3). In Nederland was slechts een klein percentage (ruim 5%) van de onderzochte patiënten geïnfecteerd met deze hybride (Hoofdstuk 2). Hieruit kunnen we concluderen dat infecties met 'serotype AD hybriden' vaker voorkomt in Mediterraan Europa in vergelijking tot het Noordwesten van Europa (Hoofdstuk 2, 3 en 4). Zuid-Amerikaanse en Aziatische studies tonen aan dat infecties met *C. neoformans* var. *neoformans*, en de hybride van deze soort met *C. neoformans* var. *grubii*, op deze continenten een ondergeschikte rol spelen. De herkomst van de 'serotype AD hybride' *Cryptococcus*-infecties in Mediterraan Europa is onbekend, het vinden van de infectiebron kan een belangrijke bijdrage leveren om het hoge percentage *C. neoformans* var. *neoformans* in deze regio te verklaren.

Tot dusver zijn er nog geen *C. neoformans* var. *neoformans* stammen op grote schaal getypeerd. Multi-Locus Sequence Typing (MLST) is hiervoor een geschikte techniek maar is een tijdrovende en dure aangelegenheid, vooral wanneer het de zogenaamde 'serotype AD hybriden' betreft omdat hierbij het gevaar van dubbele allelen om de hoek komt kijken. Een specifieke microsatelliettypering voor *C. neoformans* stammen met een serotype D achtergrond is kortgeleden opgezet en is succesvol toegepast op een set Nederlandse stammen. De resultaten van deze Nederlandse studie zijn veelbelovend, en deze techniek kan ook succesvol worden toegepast op *C. neoformans* var. *neoformans* stammen afkomstig van andere geografische locaties (Hoofdstuk 2). Microsatelliettypering heeft aangetoond dat het typeren van hybriden tussen soorten, zoals de 'serotype AD hybriden' veel minder bewerkelijk is dan MLST, en de serotype A en D achtergrond van hybride stammen kan daarnaast gemakkelijk worden aangetoond met microsatelliettypering (Hoofdstuk 2).

Microsatelliettypering toegepast op *C. neoformans* var. *grubii* heeft aangetoond dat deze typeringstechniek een uitermate goede techniek is om stammen van elkaar te onderscheiden, alsmede om de epidemiologische samenhang te bestuderen. Daarom is deze techniek toegepast op de Nederlandse collectie *C. neoformans* stammen met een serotype A achtergrond. Dit resulteerde in de observatie dat de genetische diversiteit in Nederland groter was dan die in Cuba (Hoofdstuk 2). Één of meer van de volgende redenen

kunnen de oorzaak van de grotere genetische diversiteit zijn binnen de Nederlandse *C. neoformans* populatie. Ten eerste, de tijdspanne van de Nederlandse studie was langer. Ten tweede, de opbouw van de Nederlandse populatie is gekarakteriseerd door een hoge immigratie van individuen uit de voormalige Nederlandse overzeese gebieden, alsmede immigranten uit Noord-Afrika en Oost-Europa. Het is mogelijk dat deze immigranten een latent waren geïnfecteerd met *C. neoformans* waarbij de infectie zich nog niet openbaart (een zogenaamde subklinische infectie), en dit kan hebben geleid tot een hogere genetische diversiteit in de Nederlandse *C. neoformans* populatie. Ten derde, het is recentelijk aangetoond dat *C. neoformans* in het menselijk lichaam zich door middel van genetische mechanismen een veranderende genetische populatie kan vormen. Dus een hieruit vloeiende mogelijkheid is dat subklinische infecties worden geactiveerd als de patiënt een verzwakte weerstand krijgt, door ziekte of door medicatie, waardoor *C. neoformans* cellen in het menselijk lichaam onderling interacties aangaan die de genetische diversiteit verhogen. Geconcludeerd mag worden dat microsatelliettypering een veelbelovende methode is om de dynamiek en structuur van *C. neoformans* var. *grubii* en var. *neoformans* populaties te bestuderen.

Het testen van schimmeldodende middelen op *C. neoformans* stammen is met regelmaat uitgevoerd, en inmiddels zijn er vele studies over gepubliceerd. De ontwikkeling van nieuwe schimmeldodende middelen, de zogenaamde nieuwe triazolen, en de gerapporteerde gevallen waarbij *C. neoformans* resistent blijkt te zijn tegen schimmeldodende middelen, geven aan dat het belangrijk blijft om de gevoeligheid van *C. neoformans* tegen deze middelen te blijven bepalen. Een wereldwijde studie heeft aangetoond dat minder dan 1% van de geteste *C. neoformans* stammen resistent is tegen de drie klinisch meest gebruikte middelen amphotericine B, 5-flucytosine en fluconazole. De Kroatische, Nederlandse en Spaanse studies die in dit proefschrift zijn beschreven tonen aan dat in deze landen resistentie tegen deze schimmeldodende middelen nog geen probleem is (Hoofdstuk 2, 3 en 4). Diverse recentelijk gepubliceerde studies, inclusief die in dit proefschrift, tonen aan dat posaconazole, voriconazole en isavuconazole (de nieuwe triazolen) veelbelovende kandidaten zijn om de conventionele middelen te vervangen, met name omdat deze nieuwe schimmeldodende middelen een laag toxisch effect hebben en in een lagere concentratie eenzelfde effectiviteit vertonen.

Kroatische en Nederlandse *C. neoformans* var. *grubii* en 'serotype AD hybride' stammen bleken minder gevoelig te zijn tegen fluconazole dan *C. neoformans* var. *neoformans* stammen. Daarnaast werd aangetoond dat *C. neoformans* var. *neoformans* stammen al bij lagere concentraties amphotericine B werden gedood (Hoofdstuk 2 en 4). Geen van de drie epidemiologische studies in dit proefschrift kon echter een verband aantonen tussen de klinische achtergrond van de patiënt versus de uitkomst van de gevoeligheidsbepalingen met de schimmeldodende middelen (Hoofdstuk 2, 3 en 4).

Sectie II. *Cryptococcus gattii*: genotypische diversiteit en gevoeligheid tegen schimmeldodende middelen

Tot een decennium geleden werd *Cryptococcus gattii* slechts zelden aangetroffen als de veroorzaker van cryptococcosis bij personen die leefde buiten de endemische gebieden in de tropische en subtropische klimaatzones van Centraal-Afrika, Noord-Australië en Zuid-Amerika. Dit is echter veranderd door de plotselinge uitbraak van *C. gattii* in het gematigde klimaat van Vancouver Island (British Columbia, Canada). Vanuit hier heeft de uitbraak zich de afgelopen jaren verder verspreid naar het vasteland van Canada en de Pacific Northwest van de Verenigde Staten. Een verhoogde incidentie van *C. gattii* in andere niet-endemische gebieden is waargenomen. Dit toont aan dat *C. gattii* mogelijkerwijs een groter verspreidingsgebied heeft dan werd verondersteld.

Cryptococcus gattii wordt in toenemende mate aangetroffen in Europa. Dit was voor ons de reden om een epidemiologische studie op te zetten om *C. gattii* en atypische *C. neoformans* stammen, geïsoleerd uit Europese patiënten, dieren en uit het milieu, te typeren. Dit resulteerde in een set van ruim honderd *C. gattii* stammen die werden bestudeerd met behulp van de moleculair biologische technieken Amplified Fragment Length Polymorphism (AFLP) fingerprinting en Multi-Locus Sequence Typing (MLST). De genetische samenhang tussen Europese *C. gattii* stammen en die uit andere wereldwijde locaties werd onderzocht door de toevoeging van een grote, reeds gepubliceerde, dataset (Hoofdstuk 5).

De oudst bekende beschreven *C. gattii* infectie is beschreven in 1896 en betrof een ogenschijnlijk gezonde patiënt uit Lille, Frankrijk. Sindsdien zijn *C. gattii* slechts zelden beschreven en indien een *C. gattii* werd vastgesteld was dit meestal de reden om het ziektegeval te publiceren in wetenschappelijke literatuur. Sinds een decennium is er echter een toename in het aantal Europese *C. gattii*-infecties waarneembaar. Het was echter de vraag of deze toename te wijden was aan import van *C. gattii*-infecties door toerisme en immigratie, of door dat *C. gattii* in het Europese milieu voor komt. Recente studies hebben aangetoond dat *C. gattii* zich voor vele jaren schuil kan houden in de gastheer voordat symptomen van de infectie zich voordoen (Hoofdstuk 11). De huidige MLST-studie toont aan dat klinische *C. gattii* stammen, geïsoleerd uit patiënten woonachtig in het Noordwesten van Europa, hoofdzakelijk zijn opgelopen door toerisme en immigratie van en uit endemische gebieden. Dit terwijl de meeste veterinaire en klinische *C. gattii* stammen uit het Mediterrane gebied gekoppeld konden worden aan *C. gattii* stammen die werden geïsoleerd uit het milieu (Hoofdstuk 5 en 6).

In Spanje zijn de afgelopen jaren diverse kleine *C. gattii* uitbraken vastgesteld bij geiten. Deze infecties werden veroorzaakt door stammen die behoorde tot het genotype AFLP4. Ditzelfde genotype werd ook aangetroffen nadat de leefomgeving van een Italiaanse patiënt was onderzocht. De recente observatie van veterinaire en ogenschijnlijke autochtoon opgelopen klinische infecties was de reden om in Spanje het milieu te

onderzoeken om de bron van de infecties te achterhalen (Hoofdstuk 6). De carobboom (*Ceratonia siliqua*) bleek regelmatig positief te zijn voor de aanwezigheid van *C. gattii*, deze boomsoort is endemisch in Mediterraan Europa en wordt hoofdzakelijk aangetroffen in Spanje. In het verleden werd gedacht dat alleen *Eucalyptus* soorten de natuurlijke bron van *C. gattii*-infecties waren, dit bleek echter een verkeerde veronderstelling gezien het feit dat enkele grote studies *C. gattii* aantroffen in allerlei boomsoorten behalve *Eucalyptus*. Dit bleek ook het geval te zijn bij deze Spaanse studie, slechts één *Eucalyptus* boom werd positief bevonden (Hoofdstuk 6).

De *C. gattii* stammen die werden geïsoleerd uit de Spaanse bomen werden met behulp van AFLP en MLST vergeleken met de stammen afkomstig uit patiënten en dieren. Hieruit werd geconcludeerd dat de overgrote meerderheid van deze stammen genetisch nauw aan elkaar verwant waren. Dit is een zeer sterke suggestie dat de Spaanse patiënten en dieren de infectie ook in Spanje hebben opgelopen. Interessant genoeg konden *C. gattii* stammen uit het Noordwestelijke en Westelijke deel van Spanje niet van elkaar worden onderscheiden, dit terwijl de carobboom daar niet voor komt. Dit toont aan dat de ecologische bron van Spaanse *C. gattii* stammen nog niet gevonden is (Hoofdstuk 6).

Bijna alle Mediterrane *C. gattii* stammen vormen een afzonderlijk cluster wanneer er een fylogenetisch analyse wordt uitgevoerd op de MLST-data. Dit duidt op een klonale populatiestructuur, ondanks het feit dat er verschillende mating-typen (**a** en α) voorkomen bij deze set *C. gattii* stammen (Figuur 2 in Hoofdstuk 5). De aanwezigheid van beide mating-typen toont aan dat sexuele recombinatie tot de mogelijkheden behoord. Een gelijke observatie is recentelijk waargenomen in een *C. gattii* populatie uit Papua Nieuw Guinea. Hier werd gesuggereerd dat inteelt tussen genetisch nauw verwante stammen voorkomt maar dat er ogenschijnlijk geen verstoring is van de klonale populatiestructuur. Het is mogelijk dat dit eveneens een rol speelt in de Mediterrane *C. gattii* genotype AFLP4 populatie. Het blijft echter opmerkelijk dat alle mating-type **a** stammen afkomstig zijn uit Italië terwijl mating-type α stammen alleen werden aangetroffen in Spanje, dit suggereert dat er een geografische barrière is (Hoofdstuk 5 en 6).

Recombinatie werd ook aangetoond in andere genotypische clusters van het *C. gattii*-complex, en komt overeen met recente waarnemingen uit andere studies (Hoofdstuk 5). Naast de reguliere recombinatie tussen mating-type **a** en α stammen is ook recombinatie aangetoond tussen stammen met hetzelfde mating-type. Dit mechanisme van recombineren is gesuggereerd als de drijvende kracht achter de *C. gattii* uitbraken in Noord-Amerika (Hoofdstuk 8). Signalen van recombinatie tussen stammen met hetzelfde mating-type worden echter zo verborgen, dit is een mogelijke verklaring voor de ogenschijnlijke klonale populatiestructuur in Mediterraan Europa. Dit is een sterke suggestie dat recombinatie hier wel voorkomt maar dat het onzichtbaar blijft, zoals geopperd is in diverse Australische studies (Hoofdstuk 5).

In Europa worden *C. gattii* stammen met het genotype AFLP4 alleen aangetroffen in het milieu van het Mediterrane gebied, wat kan duiden op geografische obstakels zoals het klimaat dat er lokaal heerst of de aanwezigheid van bergachtig gebied zoals de Pyreneeën en de Alpen. Dit wordt verder bevestigd door het feit dat in Noordwestelijk Europa voornamelijk *C. gattii*-infecties voorkomen die kunnen worden gekoppeld aan het reizen naar endemische gebieden buiten Europa. Daarnaast zijn klinische *C. gattii* stammen uit het Mediterrane gebied juist sterk verwant aan *C. gattii* stammen die geïsoleerd werden uit het Mediterrane milieu (Hoofdstuk 5 en 6).

MLST analyse van tien genetische loci, alsmede de MLST analyses gebaseerd op de genetische loci zoals gebruikt door andere onderzoeksgroepen, tonen aan dat het *C. gattii*-complex uit vijf monofyletische clusters bestaat. Dit ondersteunt eerdere studies waarbij gebruikt werd van andere moleculair biologische technieken, zoals M13 PCR fingerprinting en AFLP fingerprinting. MLST is een waardevolle techniek om de herkomst van klinische stammen te traceren aan de hand van een referentiecollectie van milieu- en veterinaire *C. gattii* stammen. De MLST-studie beschreven in Hoofdstuk 5 toont aan dat *C. gattii*-infecties bij personen woonachtig in het Noordwestelijke deel van Europa hoofdzakelijk opgelopen zijn tijdens reizen door en naar endemische gebieden, zoals Centraal-Afrika en Zuid-Amerika. Reactivatie van zogenaamde 'slapende' *C. gattii*-infecties blijkt ogenschijnlijk vaker voor te komen dan verondersteld werd. Dit toont aan dat *C. gattii* zeer waarschijnlijk een opportunistische en niet een primaire veroorzaker van infecties is (Hoofdstuk 5 en 11).

Ondanks de opmars van *C. gattii* als klinisch relevante gist is er een gebrek aan informatie over de gevoeligheid van *C. gattii* tegen conventionele en nieuwe schimmeldodende middelen. De relatie tussen het genotype van de *C. gattii* stam en de gevoeligheid tegen een bepaald schimmeldodend middel zijn zelden bestudeerd. Als dit al is gedaan is, is er slechts een kleine set stammen bestudeerd. Dit was voor ons de aanleiding om een wereldwijde set van 350 *C. gattii* stammen te onderwerpen aan gevoeligheidstesten tegen zeven schimmeldodende middelen (Hoofdstuk 7). Deze 350 *C. gattii* stammen werden getest voor hun gevoeligheid tegen de vier conventionele middelen amphotericine B, 5-flucytosine, fluconazole en itraconazole, alsmede de nieuwe triazole verbindingen voriconazole, posaconazole en isavuconazole. Hieruit bleek dat amphotericine B, 5-flucytosine en fluconazole de laagste activiteit vertoonde, in vergelijking met de overige middelen, om *C. gattii* te doden of te remmen in zijn groeipotentieel. De waarneming dat 5-flucytosine en fluconazole de minst effectieve schimmeldodende werking vertoonde is in lijn met voorgaande studies aan zowel *C. gattii* als *C. neoformans* (Hoofdstuk 7).

In de studie, zoals beschreven in Hoofdstuk 7, behoren de meeste *C. gattii* stammen tot de genotypen AFLP4 (39%) en AFLP6 (48%), terwijl de resterende stammen behoren tot het genotype AFLP5, 7 of 10. De voorkeur in onze studie richting stammen met het genotype AFLP4 en 6 kan zijn veroorzaakt door gebruik te maken van een niet-representatieve set stammen afkomstig uit de collecties van de personen die hun

medewerking hebben gegeven aan deze studie. *Cryptococcus gattii* collecties bestaan vooral uit stammen afkomstig van goed bestudeerde populaties in Noord-Amerika en Australië. Stammen uit deze twee populaties zijn veelal genotype AFLP4 of 6, een trend die echter ook waarneembaar is bij de minder goed bestudeerde Afrikaanse, Aziatische, Europese en Zuid-Amerikaanse populaties. In de huidige studie is onderzocht of de genetische achtergrond van *C. gattii* stammen en de geografische herkomst van invloed is op de uitkomst van gevoeligheidsbepalingen. Hieruit bleek dat klinische *C. gattii* genotype AFLP6 stammen een significant ($P<0.05$) hogere concentratie aan schimmeldodende middelen konden verdragen dan klinische stammen met het genotype AFLP4. Een opvallende waarneming was tevens dat *C. gattii* genotype AFLP4 bij veel lagere concentraties 5-flucytosine (1,401 µg/ml) en fluconazole (2.467 µg/ml) afgeremd werd in zijn groei, terwijl deze concentraties veel hoger waren om eenzelfde effect te bereiken bij *C. gattii* genotype AFLP6 stammen (respectievelijk 4,961 en 5,638 µg/ml). Fluconazole en 5-flucytosine vertoonde de minste activiteit om *C. gattii* af te remmen in zijn groei. Itraconazole, dat tot voor kort werd gebruikt om cryptococcosis te behandelen, bleek in de huidige studie effectiever te werken tegen *C. gattii* dan werd gerapporteerd door andere onderzoekers. Isavuconazole, posaconazole en voriconazole laten een uitstekende werking zien om *C. gattii* in zijn groei af te remmen, ongeacht het genotype van de stam, en zelfs tegen stammen die tegen hogere concentraties fluconazole bestand waren. Wij concluderen hieruit dat amphotericine B, itraconazole, posaconazole, voriconazole en isavuconazole de schimmeldodende middelen zijn die het actiefst de groei van *C. gattii* kunnen remmen. Isavuconazole, dat aantrekkelijke farmacologische en toxicologische eigenschappen heeft, kan een welkome aanvulling zijn aan de set schimmeldodende middelen die de clinicus heden ten dagen ter beschikking heeft om cryptococcosis te bestrijden (Hoofdstuk 7).

Sectie III. Hypervirulentie van *Cryptococcus gattii* – de Vancouver Island uitbraak

Gedurende de afgelopen decennia zijn er verschillende uitbraken geweest met schimmels die ziekte veroorzaakten bij mensen, zoogdieren, reptielen en insecten. De meeste van deze uitbraken werden veroorzaakt door voorheen onbekende schimmelsoorten, zoals *Geomyces destructans* dat de oorzaak is van het 'white-nose-syndrome' bij vleermuizen, *Batrachochytrium dendrobatidis* dat bekend staat als de 'frog-killing fungus', *Nosema ceranae* dat is betrokken bij het massaal uitsterven van honingbij-kolonies, en *Cryptococcus gattii* dat in Noord-Amerika, Zuid-Amerika en Westelijk-Australië de oorzaak is van uitbraken waarbij vele honderden mensen en dieren ziek werden.

Cryptococcus gattii kwam in de belangstelling te staan door een uitbraak in het gematigde klimaat van Vancouver Island (British Columbia, Canada). Deze uitbraak verspreidde zich verder naar het vasteland van Canada en de Pacific Northwest van de Verenigde Staten. De oorzaak van deze uitbraak is een genotype van *C. gattii* dat voorheen zeer zelden werd waargenomen en dat bekend staat als *C. gattii* genotype AFLP6. Andere recente studies hebben aangetoond dat dit genotype eveneens de oorzaak was van een *C. gattii* uitbraak onder papegaaiachtige in Brazilië, een uitbraak bij een kudde van ruim 100 schapen in Westelijk-Australië, en een verhoogde cryptococcosis-incidentie bij kinderen in de provincie Pára (Brazilië) dat ook veroorzaakt blijkt te zijn door *C. gattii* genotype AFLP6.

Een eerste poging om de herkomst van de Vancouver Island *C. gattii* uitbraak te herleiden werd ondernomen door gebruik te maken van Multi-Locus Sequence Typing (MLST). De onderzoekers trokken uit de behaalde resultaten de conclusie dat de uitbraak werd veroorzaakt door een nieuw hypervirulent genotype dat tot stand was gekomen door recombinatie tussen twee niet-virulente Australische *C. gattii* genotype AFLP6 mating-type α stammen. Een tweede studie, dat snel na de eerste werd gepubliceerd, concludeerde dat de wereldwijde populatiestructuur van *C. gattii* genotype AFLP6 klonaal is en dat daardoor de herkomst van de uitbraak niet te herleiden was. Twee meer recentere studies wezen uit dat de herkomst van de Vancouver Island *C. gattii* uitbraak waarschijnlijk in Zuid-Amerika moet worden gezocht, maar een Afrikaanse of Europese herkomst kon door de onderzoekers niet worden uitgesloten.

Om de controversie over de herkomst van de Vancouver Island *C. gattii* uitbraak te verhelderen hebben wij een set *C. gattii* genotype AFLP6 stammen uit Afrika, Australazië, Europa, Noord- en Zuid-Amerika onderzocht. Hiervoor is gebruik gemaakt van de volgende vier moleculair epidemiologische benaderingen: i) een MLST-dataset gebaseerd op zogenaamde 'sequentie gekarakteriseerde AFLP fragmenten', afgekort tot SCAR-MLST; ii) genotypering met behulp van AFLP fingerprinting; iii) een nieuw ontwikkelde microsatelliettyperingsmethode; iv) analyse van gepubliceerde MLST-data (Hoofdstuk 8). Al deze moleculair epidemiologische benaderingen toonde aan

dat de Zuid-Amerikaanse *C. gattii* populatie de grootste genetische diversiteit en de grootste effectieve populatiegrootte vertoond in vergelijking tot Noord-Amerika en Australazië. Dit is in overeenstemming met voorgaande studies die eveneens concludeerden dat de Zuid-Amerikaanse *C. gattii* stammen de hoogste genotypische variatie vertonen.

De SCAR-MLST is vervolgens toegepast om de coalescence gen genealogie te bepalen, een methode die het mogelijk maakt om een tijdschaal voor de diversificatie van *C. gattii* genotype AFLP6 vast te stellen. Deze analyse toonde eveneens aan dat Zuid-Amerika de geografische bron is van de wereldwijde *C. gattii* genotype AFLP6 populatie (Figuur 1 in Hoofdstuk 8). Interessant was de observatie dat de oudste vertakking te herleiden was tot een *C. gattii* stam dat was geïsoleerd uit een boom in een onontgonnen gebied in het Braziliaanse Amazone regenwoud. Vanuit deze stam ontstonden twee vertakkingen met *C. gattii* stammen die een herkomst in Afrika en Zuid-Amerika hebben, terwijl een derde vertakking verder opsplitste in 28 typen, inclusief vertakkingen die de stammen herbergen die verantwoordelijk zijn voor de recente *C. gattii* uitbraken (Figuur 1 in Hoofdstuk 8). De diversificatietijd tussen de huidige stammen en de overeenkomstige ouderstam werd berekend op ongeveer 30.000 jaar, de vertakking met de stammen verantwoordelijk voor de *C. gattii* uitbraak op Vancouver Island ontstond circa 2.500 jaar geleden (vertakking H1). Andere uitbraak-gerelateerde stammen werden aangetroffen in oudere vertakkingen en ontstonden circa 9.300 jaar geleden (vertakkingen H2 en H3).

Verschillende studies, inclusief de onze, hebben beschreven dat beide mating-typen **a** en α aanwezig zijn in de Europese en Zuid-Amerikaanse *C. gattii* genotype AFLP6 populaties. Dit terwijl er geen mating-type **a** stammen zijn aangetroffen in Afrika en Noord-Amerika, het is ook pas sinds kort dat bekend is dat er mating-type **a** stammen zijn aangetroffen in Australië (Hoofdstuk 7). De aanwezigheid van beide mating-typen in de Zuid-Amerikaanse populatie suggereert dat reguliere recombinatie hier plaatsvindt. Hoe dan ook, het is aangetoond dat Zuid-Amerikaanse mating-type **a** stammen niet zo fertiel zijn als hun mating-type α tegenhanger. Daarnaast is bekend dat recombinatie tussen mating-type α stammen voorkomt en het is gesuggereerd dat dit de oorzaak is van de uitbraak met een hypervirulente *C. gattii* genotype op Vancouver Island. Een verdere analyse naar de aanwezigheid van recombinatie in de vijf gedefinieerde populaties, waarbij gebruik werd gemaakt van analyses zoals Ancestral Recombination Graph en CASS, toonde aan dat de mate van recombinatie enorm verschilde tussen de populaties. Het recombinatiesignaal dat in de Zuid-Amerikaanse populatie werd aangetroffen was onevenredig in vergelijking tot de andere vier populaties. Het feit dat de oudste vertakking in de coalescence gen genealogie (Figuur 1 in Hoofdstuk 8) een Zuid-Amerikaanse herkomst heeft, en dat beide mating-typen in deze populatie voorkomen suggereert dat de hypervirulente *C. gattii* types mogelijkerwijs zijn ontstaan uit reguliere recombinaties, en niet recombinaties tussen mating-type α stammen onderling (Hoofdstuk 8).

Wij stellen dat de geobserveerde recombinatie de drijfveer is achter de verhoogde hypervirulentie van de uitbraak-gerelateerde *C. gattii* typen. Het virulentiepotentieel van *C. gattii* werd daarom verder onderzocht met behulp van een macrofaag-assay en een muis-pathogeniciteitsmodel. Stammen met het genotype AFLP6A, afkomstig van de Vancouver Island uitbraak, werden vergeleken met stammen met het genotype AFLP6B, dat bij een eerdere studie als laagvirulent werd bestempeld. Deze verkennende studie toonde aan dat *C. gattii* genotype AFLP6A stammen virulenter zijn dan genotype AFLP6B stammen (Hoofdstuk 9). Daarnaast toonde wij aan dat er een correlatie is tussen de pathogeniciteit van *C. gattii* in muizen in vergelijking tot het potentieel van *C. gattii* om zich te vermenigvuldigen in macrofagen (Hoofdstuk 9). Echter, recente waarnemingen laten zien dat *C. gattii* genotype AFLP6 stammen die geen ziekteverschijnselen opwekken bij muizen toch in staat waren om zich in een macrofaag te vermenigvuldigen (Hoofdstuk 8). Andere studies toonde recentelijk eveneens aan dat er significante verschillen bestaan in het virulente vermogen van *C. gattii* genotype AFLP6A en AFLP6B stammen. De recente uitbraak in de Pacific Northwest van de Verenigde Staten met *C. gattii* genotype AFLP6C stammen blijkt eenzelfde virulentievermogen te hebben als de genotype AFLP6A stammen die betrokken zijn bij de uitbraak op Vancouver Island (Hoofdstuk 8).

Om het virulentiepotentieel van de stammen te ontrafelen die nochtans niet betrokken zijn bij een uitbraak hebben wij een selectie van *C. gattii* stammen getest in balb/c muizen en J774 macrofagen (Hoofdstuk 8). Dit resulteerde in de waarneming dat Zuid-Amerikaanse *C. gattii* stammen met het genotype AFLP6B, afkomstig uit de oudst bekende vertakkingen (Figuur 1 in Hoofdstuk 8) eveneens hypervirulent gedrag vertonen in beide pathogeniciteitsmodellen. Echter, deze Zuid-Amerikaanse stammen zijn niet virulenter dan de stammen die betrokken zijn bij de Noord-Amerikaanse uitbraken (Hoofdstuk 8). Hieruit concludeerde wij dat hypervirulentie niet gelimiteerd is tot de uitbraak-veroorzakende *C. gattii* types, maar dat deze hypervirulente eigenschap al aanwezig was bij oudere typen, inclusief de oudste *C. gattii* genotype AFLP6 vertakking (Figuur 1 in Hoofdstuk 8). Daarnaast werden hypervirulente en niet-virulente *C. gattii* stammen aangetroffen die genetisch niet van elkaar waren te onderscheiden, dit betrof eveneens stammen afkomstig uit Vancouver Island.

De genetische basis voor de verhoogde virulentie van *C. gattii* genotype AFLP6A uitbraakstammen versus dat van genotype AFLP6B stammen werd verder onderzocht met behulp van een expressie-micro-array waarbij waargenomen werd dat het mitochondriële genoom van *C. gattii* genotype AFLP6A stammen een sterk verhoogde activiteit vertoonde in vergelijking tot stammen met het genotype AFLP6B (Hoofdstuk 9). De betrokkenheid van mitochondriën bij een verhoogde virulentie van *C. gattii* uitbraakstammen werd daarnaast verder onderbouwd door de waarneming dat de mitochondriële structuur van uitbraakstammen tubulair van vorm is. Genen die betrokken zijn bij de regulering van de mitochondriële morfologie bleken een sterk verhoogde activiteit te vertonen bij de uitbraakstammen. Dit is een verdere onderbouwing dat de tubulaire structuur van mitochondriën van *C. gattii* genotype

AFLP6A uitbraakstammen een rol spelen in de verhoogde virulentie (Hoofdstuk 9). Echter, de rol van mitochondriën in de virulentie van schimmels blijft een onontgonnen kennisgebied, tot voor kort was een link tussen virulentie en mitochondriën alleen bekend van één andere schimmel, de basidiomycete plant pathogeen *Heterobasidion annosum*. Aangezien mitochondriën zijn betrokken bij hypoxie en de oxidatieve stress response is het aannemelijk dat de mitochondriële genomen onder zware selectiedruk staan. Daarom kunnen de vele mutaties hebben geleidt tot een verhoogde virulentie (Hoofdstuk 9). Onze bevindingen laten zien dat *C. gattii* genotype AFLP6A stammen kunnen overleven in macrofagen en dat dit waarschijnlijk wordt veroorzaakt door het vermogen om tubulaire mitochondriële structuren te vormen. Hierdoor wordt een effectiever herstelmechanisme gevormd om beschadigingen veroorzaakt door oxidatieve stress en/of hypoxie in macrofagen te herstellen (Hoofdstuk 9).

Kleine uitbraken met *C. gattii* genotype AFLP6 zijn gedocumenteerd op andere locaties, inclusief die bij een kudde schapen in Westelijk-Australië, papegaaiachtige in Brazilië en de verhoogde incidentie bij kinderen in Pára (eveneens Brazilië). Tegelijkertijd zijn er diverse kleine uitbraken geweest van *C. gattii* genotype AFLP4 in Mediterraan Europa (Hoofdstuk 6). Deze voorbeelden tonen aan dat *C. gattii* zich beweegt richting de meer gematigde klimaatzones. Interessant gegeven is dat deze uitbraken zich voordoen in zogenaamde Mediterrane klimaatzones, zoals gedefinieerd is in het Köppen-Geigen klimaatclassificatiesysteem. De hypothese dat een enkel hypervirulent *C. gattii* type een plotselinge uitbraak heeft veroorzaakt kan worden uitgesloten aangezien diverse typen zijn betrokken bij deze uitbraken (Hoofdstuk 8).

Het overgrote deel van de *C. gattii* genotype AFLP6A stammen dat betrokken is bij de Vancouver Island uitbraak zijn hypervirulent, maar hierop zijn uitzonderingen aangetroffen. Een stam dat geïsoleerd is in de jaren 1970 bij een patiënt uit Seattle (VS) bleek een matig virulentiepotentieel te hebben (Hoofdstuk 9). Een milieu-isolaat, geïsoleerd in de jaren 1990 uit een *Eucalyptus*-boom, bleek niet-virulent te zijn in zowel het muis- als de macrofaag-assay (Hoofdstuk 9). Een recent uitgevoerde studie toonde aan dat bijna 65% van de geteste bewoners van Vancouver Island en ruim 17% van de geteste mijnwerkers (getest in de periode 1982-1984), antilichamen hadden tegen *C. gattii*. Deze data tonen duidelijk aan dat de Vancouver Island *C. gattii* uitbraak alreeds voor langere tijd aanwezig was in het lokale milieu alvorens de uitbraak daadwerkelijk startte.

De opkomst van de diverse *C. gattii* uitbraken op verschillende locaties kan zijn gerelateerd aan één of meerdere recente ontwikkelingen, zoals een genetisch effect (recombinatie), of een verandering van het milieu. Het is mogelijk dat de wereldwijde klimaatsverandering een sleutelrol speelt in de opkomst van uitbraken in Mediterraan Europa, Noord-Amerika en Westelijk-Australië aangezien deze tegelijkertijd ontstonden (Hoofdstuk 8). Studies naar de snelheid van de klimaatsverandering hebben aangetoond dat gematigde en Mediterrane klimaatzones tot de groep behoren van snelst opwarmende gebieden. Dus een verhoging van de gemiddelde jaarlijkse

temperatuur kan invloed hebben op de opkomst en hevigheid van uitbraken in deze klimaatzones. Een verhoogde temperatuur en factoren direct hier aan gerelateerd, zoals neerslag, luchtvochtigheid, lucht en watertemperatuur, hebben de distributiepatronen van diverse plant- en dier-pathogene micro-organismen beïnvloed, en ligt in de lijn der verwachting dat dit ook geldt voor *C. gattii* genotype AFLP6 (Hoofdstuk 8).

In conclusie: coalescence gen genealogie heeft aangetoond dat de oudste *C. gattii* genotype AFLP6 vertakking afkomstig is uit Brazilië (Hoofdstuk 8). Ongeveer 22.000 jaar geleden verspreidde deze zich naar Afrika, gevolgd door een grote diversificatie- en recombinatiegolf, ongeveer 9.000 jaar geleden, waardoor *C. gattii* genotype AFLP6 terecht kwam in Australazië, Europa en Noord-Amerika. De meeste Zuid-Amerikaanse vertakkingen ontstonden ongeveer 6.000 tot 7.000 jaar geleden door een hoge mate van recombinatie, wat mogelijkerwijs de drijfveer is achter de genetische diversiteit op dit continent. Lange-afstandsverspreiding kan hebben bijgedragen aan de intercontinentale verspreiding van *C. gattii* genotype AFLP6. Ongebruikelijke en extreme weersomstandigheden kunnen hebben bijgedragen aan de verspreiding van micro-organismen. De verspreiding van pathogene schimmels door de lucht is niet uitvoerig onderzocht, maar het is aangetoond dat ze aanwezig zijn in stof van zandstormen, wat een verklaring kan zijn voor de globale verspreiding van *C. gattii*. Hoe dan ook, de verspreiding van *C. gattii* genotype AFLP6 blijft vooralsnog een raadsel.

Sinds de opkomst van de Vancouver Island *C. gattii* uitbraak zijn honderden voorheen gezonde mensen en dieren, die in het aangetastte gebied leven, ziek geworden. Echter, toeristen die de endemische gebieden op Vancouver Island bezoeken lopen ook gevaar. Het is diverse keren aangetoond dat toeristen gedurende korte bezoeken aan Vancouver Island een *Cryptococcus*-infectie oplopen. Dit proefschrift bevat twee rapportages van *C. gattii*-infecties bij patiënten die besmet zijn geraakt tijdens een kort verblijf op Vancouver Island (Hoofdstuk 10 en 11). Het eerste gerapporteerde geval betreft een toerist die kort na terugkeer van zijn vakantie op Vancouver Island cryptococcosis ontwikkelde. De patient was een 51-jarige HIV-negatieve Deense man met een ogenschijnlijk goed functionerend immuunsysteem, hij werd echter behandeld met een non-steroïde ontstekingsremmer voor een andere aandoening. Zes weken voordat hij zich meldde bij de arts kwam hij terug van een vakantie op Vancouver Island, dit toont de korte incubatietijd van een *C. gattii*-infectie aan waarbij de klinische verschijnselen zich openbaren (Hoofdstuk 10). In Hoofdstuk 11 wordt een 42-jarige Nederlandse patiënte beschreven die een *Cryptococcus*-infectie ontwikkelde acht maanden nadat zij gediagnosticeerd was met een autoimmuun hemolytische anemie als een eerste symptoom van systemische lupus erythematosus (SLE). Zij werd behandeld met prednisolon om de SLE te onderdrukken, dit activeerde echter de latent aanwezige *C. gattii*-infectie. Uitgebreid moleculair biologisch onderzoek, waarbij gebruik werd gemaakt van AFLP fingerprinting en MLST, toonde aan de beide patiënten waren geïnfecteerd met *C. gattii* genotype AFLP6A dat genetisch identiek was aan de stammen die betrokken zijn bij de Vancouver Island *C. gattii* uitbraak (Hoofdstuk 10 en 11). De casus van de Deense patiënt toont aan dat *C. gattii* stammen

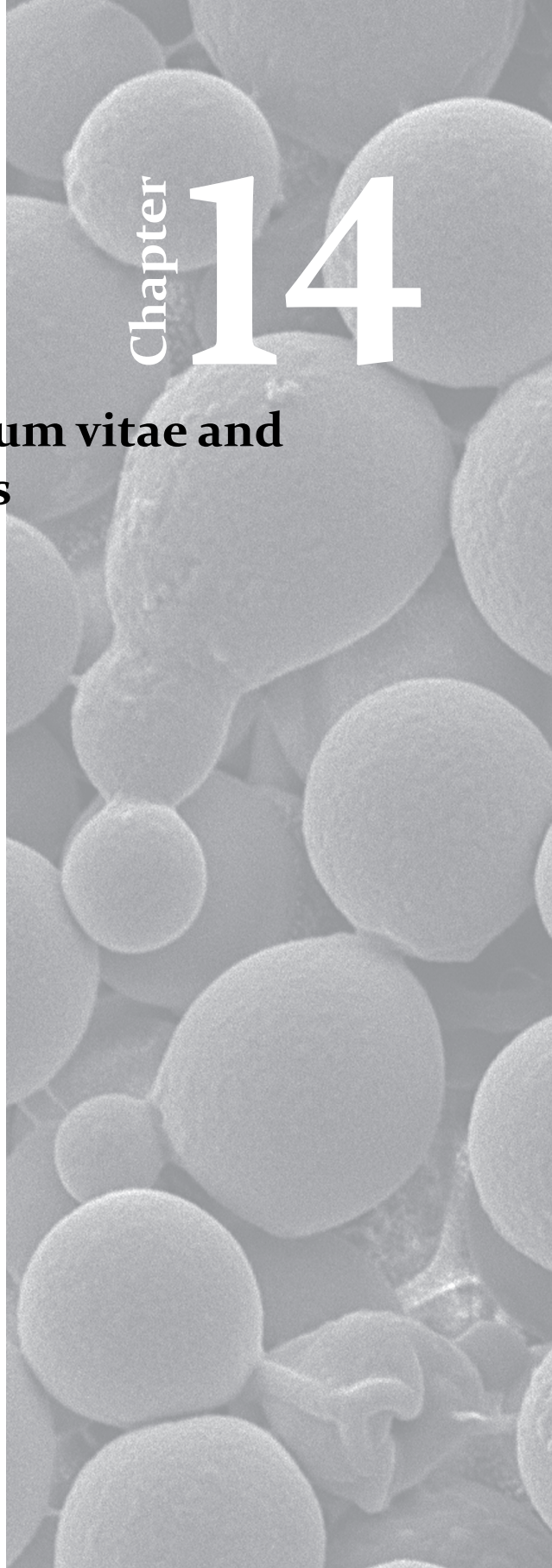
met het genotype AFLP6A een hypervirulent karakter hebben, met een incubatietijd van slechts zes weken. Daarentegen toont de casus van de Nederlandse patiënte aan dat *C. gattii* in staat is om latent in het menselijk lichaam aanwezig te zijn en pas actief te worden wanneer het immuunsysteem onderdrukt wordt. Dit is hier voor het eerst beschreven, maar was al bekend voor *C. neoformans*. Twee andere rapportages van latente *C. gattii* infecties ontberen de aanwezigheid van gedetailleerde moleculair biologische analyses. Dus de conclusies dat deze patiënten cryptococcosis ontwikkelde door een latent aanwezige *C. gattii* stam blijft uitermate speculatief. De casus van de Nederlandse patiënte, waarbij *C. gattii* latent aanwezig is bij een gezond persoon, toont aan dat bewoners van gebieden waar *C. gattii* endemisch voorkomt mogelijkerwijs ook latent geïnfecteerd zijn. Het overgrote deel van de geteste bewoners van Vancouver Island heeft antilichamen tegen *C. gattii*, daarom is het niet ondenkbaar dat zij in de toekomst cryptococcosis kunnen ontwikkelen als een resultaat van een immuun-onderdrukkende therapie (Hoofdstuk 11).

Slotopmerkingen

Dit proefschrift heeft ondersteunend bewijs aangeleverd dat de epidemiologische verschillen tussen *C. neoformans* en *C. gattii* minder strikt zijn dan voorheen werd verondersteld. Echter, fylogenetische analyses die gebaseerd zijn op datasets verkregen met behulp van verschillende moleculair biologische technieken, tonen aan dat beide gistsoorten bestaan uit monofyletische clusters die mogelijkerwijs gecorreleerd zijn aan de immuunstatus van patiënten, in het bijzonder binnen het *C. gattii* complex. De aanwezigheid van monofyletische clusters bij zowel *C. neoformans* en *C. gattii* pleiten voor een taxonomische herziening van beide soorten. Dit is momenteel echter een onderwerp van debat tussen *Cryptococcus*-onderzoekers en clinici aangezien een significant biochemisch, ecologisch en/of epidemiologisch verschil tussen de cryptische soorten nog niet is gevonden. De waarneming dat zowel *C. neoformans* als *C. gattii* gezonde personen en patiënten met een verzwakt immuunsysteem kunnen infecteren tonen aan dat er subtiele geno- en fenotypische verschillen bestaan tussen de cryptische soorten. Een soortgelijke waarneming werd gemaakt voor de *C. gattii* genotypen die betrokken zijn bij de Vancouver Island *C. gattii* uitbraak, en dit kan eveneens voorkomen bij andere genotypen. Toekomstig onderzoek zal zijn focus moeten hebben op een zoektocht naar een makkelijk toe te passen geno- of fenotypische assay om de cryptische soorten van elkaar te onderscheiden. Moderne technieken, zoals het sequentiëren van complete genomen en grootschalige populatiestudies, zullen de complexe interacties tussen het immuunsysteem van de gastheer en dat van de verschillende cryptische soorten in het *C. neoformans*/*C. gattii* complex verder ontrafelen.

Chapter 14

Nawoord, Curriculum vitae and List of publications



Nawoord

Terugkijkend op de afgelopen 4,5 jaar dat ik aan dit promotieonderzoek heb gewerkt kan ik stellen dat deze tijd voorbij is gevlogen. Achteraf gezien dan. De weg naar dit promotieonderzoek is vele jaren er voor al ingeslagen, waarschijnlijk al op een leeftijd toen ik het woord promotieonderzoek nog niet eens kon spellen maar ik ondertussen wel met een speelgoedmicroscoop in de weer was om insecten eens bij een 10× vergroting te bekijken. Dat had 'Sinterklaas' goed ingeschat toen hij het cadeau voor mij kocht. Jaren later werd de finale basis gelegd voor het laboratoriumleven toen ik in de weekenden samen met Leen aan de slag ging met chemische en microbiologische analyses in het laboratorium van de Breukelense waterzuiveringsinstallatie. Sindsdien hebben vele personen mij geënthousiasmeerd om verder te gaan in de microbiologie, met dit proefschrift als ultiem sluitstuk.

De afgelopen tien jaar heb ik in verschillende functies bij de 'gisten'-onderzoeksgroep van het Centraalbureau voor Schimmelcultures gewerkt. Teun, enorm bedankt dat je mij de kans gaf om na een stage in jou onderzoeksgroep een deeltijd HLO-opleiding te volgen terwijl ik ondertussen mijn 'moleculair biologische kunstjes' verder kon perfectioneren aan de hand van de vele *Cryptococcus* onderzoeksprojecten. Dit nawoord zou niet compleet zijn zonder jou te bedanken voor de geboden mogelijkheid om het 0-zo-begeerde promotieonderzoek uit te voeren. Hiervoor moet ik zondermeer ook Andy bedanken die als promotor het reilen en zeilen van mijn vorderingen voor en tijdens mijn promotieonderzoek in de gaten hield en mij ervan overtuigde dat er best een hoofdstuk of twee meer in het proefschrift mochten worden opgenomen.

Mijn echte carrière in de microbiologie begon echter al tijdens mijn opleiding tot microbiologisch analist waarvoor ik een afstudeerstage volgde in de onderzoeksgroep Microbiële Ecologie van het Centrum voor Limnologie. Gabriël gaf mij de haast onmogelijke stageopdracht om in vijf maanden tijd de bacteriën te kweken die kort daarvoor met behulp van moleculaire technieken waren aangetoond. De meest wilde ideeën werden uitgevoerd om deze 'onkweekbare' bacteriën als culture in handen te krijgen. Jammer dat het niet is gelukt. Wat echter wel lukte was om mij enthousiast te maken voor de moleculaire biologie. Daarnaast kreeg ik de mogelijkheid om ruim een jaar, naast mijn MLO-opleiding, mee te draaien in het moleculaire laboratorium waar Miranda, Marion en Paul mij met veel enthousiasme de meest uiteenlopende moleculaire technieken aanleerden. Hiermee was voor mij definitief de weg ingeslagen naar een toekomstig promotieonderzoek.

Mijn promotieonderzoek heeft zich voor een aanzienlijk deel afgespeeld in de laboratoria van het Canisius Wilhelmina Ziekenhuis waar ik onder toeziend oog van Jacques, Tere, Ilse en Erik aan de slag ben gegaan met gevoeligheidsbepalingen van honderden *Cryptococcus*-stammen, hoe meer stammen hoe beter de statistiek! Samen met Corné zijn er heel wat dagen besteed aan het genereren en analyseren van microsatellietdata van duizenden *Cryptococcus*-stammen (hoe zal het met de statistiek zitten vraag je je

dan af). Corné en Jacques, bedankt dat jullie mij de mogelijkheid hebben gegeven om bij het CWZ verder te werken aan mijn carrière als Medisch Moleculair Microbioloog!

Tijdens de vele jaren schimmelonderzoek bij de 'gistenclub' heb ik met vele mensen samen mogen werken, waaronder vele gastonderzoekers uit alle uithoeken van de wereld. Dit resulteerde in een verfrissende kijk op het onderzoek, al dan niet veroorzaakt door spraakverwarringen. Het spreekt voor zich dat ik mijn ex-groepsgenoten wil bedanken voor hun steun en toeverlaat gedurende de afgelopen jaren: Bart als de constante factor in de groep, Rolf die een promoverende postdoc bleek te zijn, mijn voorgangster Marjan die de weg plaveide voor mijn aandeel in de *Cryptococcus* taxonomie, de bioinformatica-Brazilianen Eiko en Saulo, en Kenneth die met zijn onderzoek aan de septal pore caps de veelzijdigheid van de werkgroep complementeerde.

The versatility of our research group was visualized by the large number of visiting researchers from abroad who mainly worked on projects to determine the yeast diversity, ranging from the Colombian rainforest to Italian glaciers. Nevertheless, they could not escape from my enthousiasm for cryptococcal research. I would like to thank the persons with whom I have worked together on diverse projects, even on non-*Cryptococcus* projects (unbelievable but true): Aída who made us slightly jalous with the stories about her Colombian rainforest field trips, Anna who frequently prepared nice food to celebrate another important Polish day that needs to be commemorated, Benedetta who nearly killed me after I boiled the pasta thirty seconds longer than necessary according to unwritten but official Italian pasta-laws, Ning the silent Thai power behind a large *Cryptococcus*-study, the Colombian Carlos 'Loco' and the Spanish Carlos 'Litros' with their meaningful nicknames, Retno who escaped several times her hometown Jakarta to learn more about *Cryptococcus* while practising Dutch, hard-working Weihua who confiscated my lab bench during the weeks so that I had an excuse to work during the weekends, and, finally, Hansong and Kerstin who visited our research group to work together with me on the famous cryptococcal outbreaks. I also would like to thank Daniëlle, Emilija, Françoise, Greetje, Itzhack, Jesús, Karen, Kika, Leen, Leo, Paulo, Robin and Steven for the nice and productive collaboration during the past (and for sure the forthcoming) years.

Een grote groep studenten heb ik gedurende de afgelopen tien jaar mogen begeleiden. Het spreekt voor zich dat ik met name dank verschuldigd ben aan de *Cryptococcus*-'minded' studenten Alwin, Arjan, Collin, Dave, Jeroen, Jolien, Mattijs en William die mij gedurende de afgelopen jaren hebben geholpen met het genereren een datastuwmeer. Hoewel ik bekend stond als de stagiairebeul van het instituut werd ik toch uitgenodigd door de Weertse studenten om in Weert carnaval te komen vieren. Dit aanbod kon ik uiteraard niet afslaan, vele Weerter stapavonden op de Oelemarkt volgden. Collin, Dave, Ellen en Sanne, bedankt voor de vele gezellige stapnachten (en soms zelfs complete weekenden) doorhalen in Weertse feesttenten waarbij het verdriet van mislukte experimenten vaak meer dan voldoende werd weggefeest. En als er niet gefeest werd in Weert of Utrecht dan was het wel op de route er naar toe, of er zelfs voorbij, getuigen

de ontelbare keren dat ik naar 's Hertogenbosch, Eindhoven en Maastricht afgereisd ben om de voorgaande werkweek weg te feesten, of de nieuwe te verwelkomen, samen met de 'party-animals' Gijs, Salvatore, Laura, Bart en Noviya. Daarnaast wil ik ook mijn 'niet-lab'-vrienden bedanken die na al die jaren nog niet weten wat pipetteren inhoud, en dit ogenschijnlijk graag zo willen houden. Met name Marieta wil ik bedanken voor de gezelligheid tijdens de vele jaren zwemmen van de 'zestig banen' waarbij geuitte labfrustraties veelal afgestraft werden met tien extra banen.

Al dit feesten leverde ook serieuze kost op in de vorm van ons eigen wetenschappelijke tijdschrift dat geen impact factor had, maar toch een diepe indruk achterliet: Macaroni Reviews. Het was echter geen lang leven beschoren want na een paar jaar moesten Kenneth, Gijs, Marjan en ikzelf toch echt serieus aan de slag met het schrijven van publiceerbare wetenschappelijke kost in plaats van het beschrijven van stapavonden. Wat wel een constante factor is geweest gedurende de tien jaar zijn de maandelijkse etentjes, regelmatig startend en eindigend met borrels in de Utrechtse binnenstad, met mijn CBS-matties Neriman, Tineke, Joost, Richard, Rolf en Timon. Ik vraag mij echter af hoe lang deze maandelijkse etentjes nog bekend zullen staan als 'AIO-dinners' nu er meer postdocs in de groep zitten dan promovendi!

Sinds mijn eerste werkdag als promovendus had ik schijnbaar wel de tijd om enkele keren per dag een tussenstop te maken bij de koffietafel. Naarmate één van de vele deadlines zich aandiende werd de koffiepauze wel ontzettend kort, maar toch. Desalniettemin ben ik sinds 4,5 jaar bekend komen te staan als de DTO-wannabe aangezien ik de eerste niet DTO'er was die het durfde om aan te schuiven aan een DTO-gedomineerde koffietafel. Hoe dan ook, het onderwerp was niet meer standaard *Aspergillus*, *Talaromyces* of *Penicillium*, maar er was zelfs ruimte om het woord *Cryptococcus* te laten vallen (de eerste keer dat dit gebeurde viel er een lange diepe stilte). Interessant genoeg blijkt diegene die met regelmaat '*Cryptococcus* is sooo boring!' riep toch zelf in een ver verleden heel wat onderzoek te hebben verricht aan deze gist, of niet Rob? Desondanks zijn de koffietafelmomenten voor mij uitgegroeid tot de dagelijkse rustmomenten terwijl sterke verhalen van de DTO'ers Carmo, Henk S., Jan, Jos, Martin, Mirjam, 'Turkish Delight' Neriman, Richard, Rob, Tineke, Timon, maar ook die van de andere CBS-collega's, Ad, Alexa, Henk B., Joost, Maikel, Marloes, Ronald, Sylvia en William Q, bij mij het ene oor in gingen, en regelmatig na wat opmerkingen mijnerzijds, het andere oor weer uit. Als we maar lol hebben!

En lol heb ik inderdaad gehad tijdens de schimmelcongressen in Athene, Cairns, Edinburgh, Tokyo, Turijn en Valencia (en ja, ik heb ook veel opgestoken van de wetenschappelijke presentaties!), waar ik samen met andere CBS'ers vele hilarische momenten heb meegemaakt bij de CBS-stand waar Manon en Tineke elke passerende congresbezoeker scherp in de gaten moesten houden omdat anders de complete kraam werd meegenomen. Ze hebben zelfs een fan uit India die alleen schimmelcongressen bezoekt waar zij met de CBS-stand staan. Tineke, Rolf en Ilse, ons congresbezoek aan Tokyo staat bij mij nog steeds op nummer één, jammer dat ze niet bij elk congres een

bar op de 25ste verdieping hebben om verhalen 'uit den oude doosch' op te diepen!
Het CBS-leven speelt zich niet alleen af op de eerste verdieping. Al tijdens mijn afstudeerstage wist ik feilloos de weg te vinden naar de de gistenclub op de begane grond waar Marizeth, Maudy en Wendy de gistcollectie beheren. De gistcollectie zit aan het einde van de gang en daardoor heb ik heel wat 'overleg' gehad met de collega's die in de aangrenzende kamers bivakkeerden: Arien, Arthur, Francis, IJda (mijn chauffeuse voor de talloze transfers tussen thuis en werk wanneer het OV het weer eens liet afweten), Karolina, Marjan F., Marjan V., Marlene (ik ga echt wel een keer naar Suriname), Remco, Rina, Ron, Sander, Tinie, Vincent, Trix, en de collega met de grootste werkkamer van het instituut, Thea. Bedankt voor alle hulp en gezelligheid gedurende de afgelopen jaren!

Op de eerste verdieping ben ik, naast de hierboven genoemde DTO- en 'gisten'-collega's, ook de collega's van andere onderzoeksgroepen zeer erkentelijk voor de talrijke (al dan niet wetenschappelijke) discussies: Pedro, naast CBS-directeur ook 'Chef Pizza-party', Mieke 'Ferry, heb je weer je onzichtbare labjas aan?', Ewald 'Chef Fylogenetische Analyses', Ulrike en de Zuid-Afrikanen Gavin, Lizel en Lorenzo. Van de onderzoeksgroep waar de 'minder plesante' medische gisten worden onderzocht ben ik Bert, Ela, Hamid, Kasper, Montarop en Sybren erkentelijk voor de vele discussies over moleculaire- en populatiebiologie. Ela, I would like to thank you for the numerous talks during the past years, at congresses, parties and of course in the 'binnentuin' or the molecular lab, to share thoughts about 'life as a PhD student' with the numerous positive and -unfortunately- some negative sides of it. The 'Young ISHAM'-meeting, next year, will be the crown on the many years of energy and work that we have put in this to setup the working group for young medical mycologists.

Mijn ex-CBS-kamergenoten en tevens paranimfen Richard en Timon. We blijken gewoonweg al jaren de gezelligste werkkamer van het CBS te hebben gehad, ik kan geen andere reden bedenken waarom vele collega's elke dag speciaal vanaf de koffiemachine naar onze kamer kwamen lopen voor een (al dan niet) therapeutisch gesprek. Ondanks dat zaten we ogenschijnlijk ook nog eens in de productiefste werkkamer want waar anders werken ze nog van acht tot acht? Menig succes, en zo-nu-en-dan een dieptepunt, heb ik met jullie gedeeld. Er is bovenal enorm veel gelachen, van 'foutjes' bij het uitvoeren van onderzoek tot de talrijke foute maar scherpe bureau-grappen.

Tot slot wil ik mijn directe familie bedanken voor de steun in de aanloop naar en tijdens mijn promotieonderzoek. Mijn broers en schoonzussen Ruud, Arjan, Michelle en Leonie, nu hoeven jullie niet meer naar mijn enthousiaste verhalen over promotieonderzoek te luisteren, maar de microbiologie- en reisanekdotes die blijven! Arie en Leyda, bedankt voor de steun en het vertrouwen in mij gedurende het studie- en promotietraject waardoor ik deze mijlpaal uiteindelijk heb kunnen bereiken!

Ferry

22-8-2011

Curriculum vitae

Ferry Hagen was born on February 27th 1979 in Hilversum, the Netherlands. However, the foundation for his interest in microbiology was laid on a farm near the idyllic river Vecht in the village of Loenen aan de Vecht. In 1995, after he received his MAVO-diploma from the Savornin Lohman MAVO in Hilversum, he started with a MLO-study at ROC Midden Nederland in Utrecht.

Early 1999, he received his MLO-diploma 'Microbiology' after he finished his internship at the NIOO-KNAW Centre for Limnology in Nieuwersluis. Here, he worked under supervision of Dr. Gabriël Zwart on a project to identify uncultivable freshwater bacteria. During this five-month internship his interest in the scientific side of microbiology was further fueled. In 2000, he started as a parttime research technician at the same institute to investigate the bacterial diversity in Lake IJsselmeer using various molecular techniques. After finishing this project he stayed at the Centre for Limnology to work for three months as a research technician, supervised by Dr. Paul Bodelier, on a project to determine changes in the diversity of ammonia oxidizing bacteria in flooded soils.

To obtain his MLO-diploma 'Medical Microbiology' he started in September 2001 with an internship at the CBS-KNAW Fungal Biodiversity Centre in Utrecht. During a nine-month internship, supervised by Dr. Teun Boekhout, he investigated the epidemiology of the pathogenic yeast *Cryptococcus neoformans* using several molecular techniques. This internship paved the way for a future PhD project, to reach this goal he started in September 2002 with a parttime HLO-study at the Hogeschool Utrecht in Utrecht. At the same time, he started with a five-year career as a research technician at the CBS-KNAW under guidance of Dr. Teun Boekhout. The aim of this research was to investigate the epidemiology and virulence of *C. neoformans* and its sibling *C. gattii* for which a diverse set of microbiological and molecular tools was used. In 2005, he received his HLO-diploma 'Molecular Biology' after he finished his internship at the CBS-KNAW, during this period he focused his research on the expanding *C. gattii* outbreak in Canada.

In December 2006 he started as a PhD student at the CBS-KNAW in the same research group. The research that was performed during the subsequent 4.5 years is described in this thesis. At present he holds a position as a postdoctoral researcher at the CWZ Canisius Wilhelmina Hospital in Nijmegen. In November 2011 he will start with a traineeship Medical Molecular Microbiology at the same hospital.

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